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The Journal of Biochemistry, Vol. 42, No. 6, 1955

ON THE STRUCTURE OF LYSOZYME

III. ON THE CARBOXYL-TERMINAL PEPTIDE

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(Received for publication, June 14, 1955)

In the previous reports lysozyme was characterized to possess one carboxyl-terminal lecuine (*1*), one aspartic acid, twelve asparagine and four glutamine residues in the peptide chain (*2*) by means of hydrazinolysis method. This method may be extended, further, to elucidate the carboxyl-terminal peptide of the protein. The principle of this method is as follows. On hydrazinolysing the protein partially a mixture of peptide hydrazides and peptides (C-terminal) is obtained. These are, altogether, dinitrophenylated with dinitrofluorobenzene (DNFB), and the dinitrophenyl-peptides (DNP-peptides) derived from C-terminal are separated from other DNP-peptide hydrazides by fractional extractions in the same manner as reported previously (*1*). C-terminal peptide fraction contains C-terminal DNP-amino acid and DNP-peptides of various lengths bearing the same C-terminal amino acid. These are separated on suitable chromatograms and the amino acid compositions of each peptides are determined by the following procedures: (a) After acid hydrolysis of DNP-peptide, DNP-amino acid derived from N-terminal amino acid of the peptide, is extracted and characterized by chromatography and estimated colorimetrically; (b) The residual free amino acids which compose the peptide are again dinitrophenylated, characterized and estimated in the same way.

If the following series of DNP-derivatives, DNP-A(BCD), DNP-B(CD), DNP-CD and DNP-D (A, B, C, and D denote four kind of amino acid residues, respectively, and the sequences in parentheses are not known) are found by the above mentioned procedure, it may be concluded that the sequence of the C-terminal peptide is -A-B-C-D (COOH).

The present study was designed to apply this principle to the determination of amino acid sequences of lysozyme from its C-terminal. To achieve this purpose the hydrazinolysis method to characterize C-terminal

amino acids was somewhat modified. In this paper five amino acid sequences from C-terminal of lysozyme shall be described.

EXPERIMENTAL

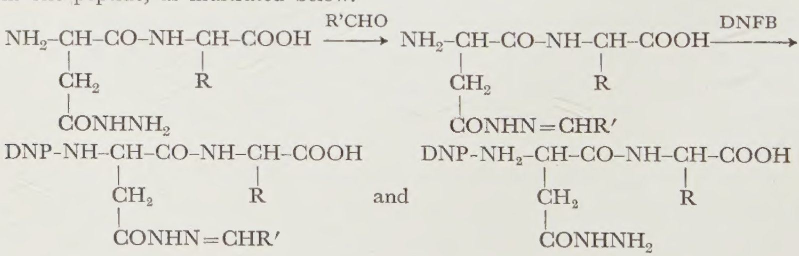
Materials—Crystalline isoelectric lysozyme was prepared in the same way as reported previously (*1*). Anhydrous hydrazine was prepared according to the method of Stähler (*3*).

Hydrazinolytic Procedures—In all partial hydrazinolyses 0.1 to 1 g. of lysozyme and about five times of anhydrous hydrazine (v/w) were used. The amount of hydrazine was enough to dissolve the protein soon. Most hydrazinolyses were made for various lengths of time with anhydrous hydrazine in a boiling water bath. On referring to the curve of the hydrazinolysis of lysozyme (*1*), the time of hydrazinolysis was selected to be 3–5 hours in order to stop the degree of hydrazinolysis at the point where C-terminal leucine would appear about 0.3–0.5 moles and the other C-terminal would remain as peptides.

Some other techniques of partial hydrazinolysis, *e.g.*, for several days at 37° in a sealed tube or some ten hours over methanol bath, were tried but found to have no advantages.

Dinitrophenylation and Extractive Procedures—The partial hydrazinolysate was poured into an watch glass and desiccated over sulphuric acid to remove excess hydrazine.

The dinitrophenylation of the hydrazinolysate and the fractional extraction of the C-terminal DNP-peptides from other di-DNP-peptide hydrazides were principally same to the procedure reported before (*1*), but in some cases some modifications were made to remove bulk of the hydrazides by condensing with benzaldehyde (*4*) or isovaleraldehyde (*5*). The pre-teratment with aldehydes has an advantage that bulk of the hydrazides is removed whereby successive analysis of C-terminal groups becomes simpler (*5*), but it is unadvisable when C-terminal peptide contains amide groups, as is the case of lysozyme. As pointed out in elsewhere (*6*), hydrazide group which is formed from amide group by hydrazinolysis combines with aldehyde by aldehyde-treatment, but on successive treatment part of the aldehyde is released from the peptide-aldehyde compound because the solution becomes alkaline and acidic by dinitrophenylation and extraction, and hence two sorts of DNP-peptide derivatives might be formed from one peptide, as illustrated below.



In fact this is found to be true in the present study, so that the aldehyde treatment was avoided in later experiments. As an example, a procedure starting from 1 g. of lysozyme will be described in the following lines. The partial hydrazinolysate was dissolved in 100 ml. of water and added with 5 g. of sodium bicarbonate. Five ml. of dinitrofluorobenzene in 200 ml. of ethanol was added to the solution and the mixture was shaken for 2 hours in a dark room. After removing the bulk of alcohol under reduced pressure at room temperature (not more than in one hour), 100 ml. of water and 70 ml. of 2 *N* hydrochloric acid were added to the reaction mixture. Then the solution was extracted with 200, 50, 30 ml. of ethyl acetate, by which almost all of the DNP-compounds were taken out. After washing twice with 100 ml. of water, the ethyl acetate solution was extracted four times with 200 ml. of 2 per cent sodium bicarbonate solution, into which DNP- compounds bearing free carboxyl group were transferred. The bicarbonate solution, after being washed twice with 200 ml. of ethyl acetate, was acidified with 130 ml. of 2 *N* hydrochloric acid and re-extracted with 100, 100 and 50 ml. of ethyl acetate. The final ethyl acetate solution which contained C-terminal peptides was evaporated *in vacuo* and analysed as described below.

Analysis of the Mixture of C-Terminal DNP-peptides—As preliminary experiments, the whole mixture of C-terminal DNP-peptides was analysed for the purpose to confirm

TABLE I

Scheme of Preliminary Experiments

Lysozyme (1 g)

↓ hydrazinolysis at 100° for 4 hrs., evaporation excess N_2H_4 *in vacuo*, dinitrophenylation, fractional extraction.

Mixture of C-terminal DNP-peptides

↓ fill up to 100 ml. with ethyl acetate.

1 ml.	*ca.* 1 ml.	*ca.* 1 ml.	Remainder
↓ EtAc↑ N_2H_4 100° 9 hrs. N_2H_4↑ dinitrophenylation, fractional extraction	↓ EtAc↑ 6 *N*HCl 100° 5hrs. extraction of DNP-amino acids	↓ EtAc↑ 6*N*HCl 100° 10hrs. dinitrophenylation extraction of DNP-amino acids	↓ Determination of C-terminal peptides.
C-terminal DNP-amino acid ↓ chromatography DNP-leucine 0.304μM (46%) Di-DNP-aspartic acid-α-hydrazide 0.073μM (11%)	N-terminal DNP-amino acids chromatography Leu>Asp>Glu>Ser>Ala>Gly (DNP-derivatives)	DNP-amino acids ↓ chromatography Leu>Asp>Ala>Gly>Glu>Ser (DNP-derivatives)	
(C-terminal amino acid of C-terminal DNP-peptide mixture)	(N-terminal amino acids of C-terminal DNP-peptide mixture)	(Amino acid composition of C-terminal DNP-peptide mixture)	

whether this was contaminated with non-C-terminal peptides (*e.g.* any DNP-peptides derived by secondary hydrolysis of the peptide hydrazides). For these purposes the characterization of N- and C-terminal aimno acids and of the constituent amino acids of the peptides were carried out.

The characterization of C-terminal amino acid of the peptide mixture was done according to the method reported previously (*1*). The C-terminal DNP-peptide mixture, which was obtained from 1 g. lysozyme after 4 hours' hydrazinolysis, was dissolved in 100 ml. of ethyl acetate, and 1 ml. of the solution (coresponding to 10 mg. (0.666 μM) of lysozyme) was evaporated *in vacuo*. (The residual solution was used to the determination of C-terminal peptide.) This was hydrazinolysed again with 0.5 ml. anhydrous hydrazine for 9 hours at 100°. Treating the hydrazinolysate in the same way as described previously (*1*), C-terminal amino acid fraction was obtained. On chromatographing and estimating colormetrically, 0.304 μM (46 per cent) of DNP-leucine and 0.073 μM (11 per cent) of di-DNP-aspartic acid-α-hydrazide were identified, and none of other DNP-compounds was detected. This result indicates that the C-terminal peptide mixture is composed of peptides which has leucine as sole C-terminal amino acid, and no other by-reactions, such as hydrolysis during hydrazinolysis or successive treatment, occurred. The di-DNP-aspartic acid-α-hydrazide, which may be supposed to be derived from inner aspartyl peptide (*2*), is not always derived from C-terminal peptide. Similar to the ε-DNP-lysine peptides in the case of the determination of N-terminal peptide (*7*), it is possible that some peptide hydrazides of non-C-terminal bearing free ω-carboxyl group of acidic amino acids might contaminate the C-terminal peptide mixture. However, the presence of such peptide hydrazides in the mixture could not be confirmed nor excluded unless they were separated directly.

The N-terminal amino acids and the amino acid composition of the whole C-terminal DNP-peptide mixture were examined in the following way. The C-terminal peptide mixture was hydrolysed with 6 N hydrochloric acid at 100° for 5 hours, and after diluting by equal volume of water, the hydrolysate was extracted with ethyl acetate. The ethyl acetate extract was evaporated *in vacuo*, and chromatographed on silica gel columns. Thus, DNP-derivatives of leucine, aspartic acid, glutamic acid, serine, alanine and glycine were detected as N-terminals. Although the amounts of these amino acids were varied with the conditions of hydrazinolysis and hydrolysis, and precise estimations of them were not done, the amounts of these amino acids were in the order as described above from much to less.

On the other hand the whole hydrolysates were evaporated *in vacuo*, the mixture was again dinitrophenylated and chromatographed. Thus the same amino acids were detected as the constituents of the C-terminal peptide mixture, but the amounts of them were in the following order : leucine>aspartic acid>alanine>glycine>glutamic acid>serine.

These facts might suggest that the C-terminal peptide is composed of these amino acids having leucine as C-terminal and the other amino acids cited above are sequent from the C-terminal according to the amount of each amino acid, but the fact is not so simple because the aspartic acid of non-C-terminal might be included in the C-

terminal mixture, and further the peptide hydrazide including aspartic acid residue might contaminate the mixture. However, it might at least indicate that the C-terminal peptide is composed of amino acids which are, if not of all, among the amino acids cited above.

Chromatographic Procedures—To separate C-terminal DNP- peptides each other, column chromatography using silica gel (*7*, *8*) and " Hyflo Supercel " (*9*), respectively, was used. In some experiments elution chromatography using silica gel was also used. The latter and Hyflo Supercel column chromatography were found to be suitable for bulk separation of large amount of DNP-peptide mixture.

Solvents used in the silica gel chromatography were as follows :

n-Butanol	Chloroform	10% Acetic acid
0	100	100
3	100	100
10	100	100
20	100	100

In the case of Hyflo Supercel columns solvents used were as follows:

n-Butanol	Chloroform	Buffer	
5	95	*M*/10 phosphate	pH 6.4
10	100	,,	,,
20	80	,,	,,
50	100	*M*/5 Acetate,	pH 4.3
Methylethylketone			
100	20	*M*/10 phosphate,	pH 6.2

Silica gel was prepared according to Consden *et al.* (*10*), but the aging of the gel was made in water instead of in dilute hydrochloric acid. Thus silica gel having a strong adsorptive power was obtained. This was convenient for the separation of DNP-peptides, because solvents containing much *n*-butanol in chloroform could be used, which dissolved more DNP-peptides than chloroform alone, but 5 to 10 per cent acetic acid had to be used as stationary phase instead of water. Some solvent systems other than chloroform were tried but none of them gave satisfactory results. Although silica gels which have similar R values described by Sanger (*8*) were obtained, bands of DNP-compounds became broad and could not be used.

Columns were 10 to 20 cm. long and their diameters were 7 to 50 mm. The lengths and the diameters were varied according to the amounts and the sorts of samples.

When a mixture of C-terminal DNP-peptides was subjected to the chromatography, several bands were separated on the column. Each bands were cut off, eluted with methanol, evaporated *in vacuo*, and further subjected to another column chromatography. Thus, each DNP- peptides were purified by chromatographing five to seven times with various solvents. It was found very often that a component, which was regarded as pure by several times chromatography, was proved to be inhomogeneous, because two or more DNP-amino acids (N-terminal of the peptide) were detected from it after acid

hydrolysis. Therefore, each component was chromatographed 2 times more after getting the unique band on a column.

On eluting DNP-compounds with methanol and evaporating the solution *in vacuo*, the residual DNP-compounds were found very often to become insoluble in developing solvents, so that the residue was taken up by 1 per cent sodium bicarbonate solution and extracted with ethyl acetate after acidification. Evaporating the ethyl acetate, the residue became soluble in any developing solvents.

Analysis of the Isolated DNP-peptides—The procedure for anaylsing the DNP-peptides isolated was similar to that for N-terminal DNP-peptides (*11*, *12*). The analysis was done by hydrolysing each peptide completely, extracting the DNP-amino acid thus released (N-terminal amino acid of the peptide), dinitrophenylating the remaining free amino acids which constituted the non-N-terminal amino acids of the peptide, and finally identifying the DNP-amino acids thus produced.

The hydrolysis was made by heating at 110° in sealed tubes with 6 *N* hydrochloric acid for some ten hours. The cooled hydrolysate was diluted with equal amount of water and extracted three times with ethyl acetate in order to remove the N-terminal amino acid. After the aqueous layer was evaporated to dryness *in vacuo*, the residue was taken up with sodium bicarbonate solution (0.1 g. $NaHCO_3$/ml.) and twice the volume of an ethanolic solution of DNFB (5 per cent) was added. After shaking for two hours, the reaction micture was transferred to a separatory funnel with ten volumes of water, extracted while still basic four times with ether to remove excess DNFB, acidified, and extracted with ethyl acetate to remove the DNP-amino acids.

Both the DNP-amino acid solutions obtained by the extraction of the hydrolysate and by the successive dinitrophenylation of remaining amino acids were evaporated *in vacuo*, and chromatographed on silica gel columns, and all the DNP-amino acids were identified and estimated colorimetrically in 1 per cent sodium bicarbonate solution.

What is peculiar to this procedure is that the asparaginyl or glutaminyl residues in C-terminal peptide can be distinguished from aspartyl or glutamyl residues. By the partial hydrazinolysis and successive dinitrophenylation, amide groups change to dinitrophenylhydrazide groups, *i.e.*,

$$\begin{array}{ccccc}
\text{-NH-CH-CO-} & \xrightarrow{NH_2NH_2} & \text{-NH-CH-CO-} & \xrightarrow{\text{DNFB}} & \text{-NH-CH-CO-} \\
| & & | & & | \\
(CH_2)_n & & (CH_2)_n & & (CH_2)_n \\
| & & | & & | \\
CONH_2 & & CONHNH_2 & & \text{CONHNH-DNP}
\end{array}$$

This –CONHNH–DNP group has reddish brown colour in alkaline solution while it become yellow in acidic solution. This phenomenon can be applied briefly to distinguish the amide groups in C-terminal peptide, although the procedure reported before (*2*) can be applied to each isolated C-terminal DNP-peptide derivatives if they are available enough.

RESULTS

The results obtained by the treatment mentioned above shall be described hereinafter. It must be mentioned, however, that being the separation of C-terminal peptides from each other rather incomplete, the clear-cut data were picked up from several runs of experiments.

The C-terminal DNP-peptide mixture was separated on chromatograms into several bands, two of the typical chromatograms being illustrated in Fig. 1 a and 1 b, which were obtained on the sample ex-

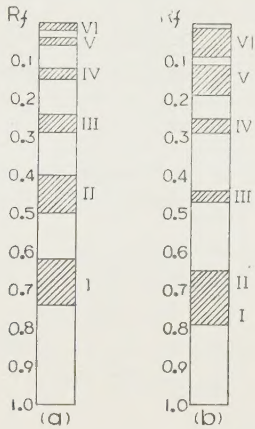


FIG. 1. Chromatograms of *C*-terminal DNP- peptides of lysozyme
(a) Adsorbent, Hyflo Supercel ; solvent, *n*-butanol-chloroform-phosphate buffer (*M*/10, pH 6.4) (1:4:5:)
(b) Adsorbent, silica gel ; solvent, *n*-butanol—chloroform—10% acetic acid (3:100:100)

cluding aldehyde-treatment. If pre-treated with aldehydes before dinitrophenylation gave more complicated chromatograms. The band which remained at the top of the columns was separated further into a few bands by another columns. Some other faint bands were sometimes detected, but did not subjected further study on them.

As shown in Fig. 1 six main components were obtained from the C-terminal DNP-peptide mixture of lysozyme. The amounts of these components varied depending on the time of hydrazinolysis and successive treatments, but the component I which corresponded to DNP-leucine was most eminent, II and VI the next, and III, VI and V were

little. Several other components, most of which moved slowly on chromatograms were not subjected to further study. After these components were chromatographed several times, they were analysed their N-terminal amino acids and amino acid compositions. Thus the six components were characterized as shown in Table II. Their quantitative data are shown in Fig. 2. The component III was so little that it could not be purified to be analysed in a clear-cut result and hence this component was only supposed its amino acid sequence qualitatively.

TABLE II

Analysis of C-Terminal Peptide

Component No.	Hydrolysate		Original peptide
	N-terminal	Amino acid composition	
I	Leu	(Leu)	Leu
II	Asp	(Asp), Leu	Asp(NH_2)-Leu
III*	Ala	(Ala), Asp, Leu	Ala-Asp(NH_2)-Leu
IV	Gly	(Gly), Ala, Asp, Leu	Gly-Ala-Asp(NH_2)-Leu
V	Asp	(Asp)	Asp (in peptide)
VI	Asp	Asp, Gly, Ala, Leu	Asp-Gly-Ala-Asp(NH_2). Leu or Asp(NH_2)-Gly. Ala-Asp(NH_2)-Leu

* The analysis of this component was not clear-cut owing its small quantity.

Combining the results cited in the Table II, it is concluded that the C-terminal peptide of lysozyme is asparaginyl (or aspartyl)-glycyl-alanyl-asparaginyl-leucine. The reason that the carboxyl group of the aspartyl residue before leucine is not free but amide is as follows. When the component corresponding to the DNP-dipeptide was dissolved in sodium bicarbonate solution, the colour of the solution became not yellow as in the case of usual DNP-peptides, but brown red, the phenomenon being characteristic to DNP-hydrazides. In addition, on hydrolysing the DNP-peptide with hydrochloric acid, an yellow band corresponding to dinitrophenylhydrazine was detected beside the band of DNP-aspartic acid. Thus it was supposed that the DNP-peptide derivative had the following constitution :

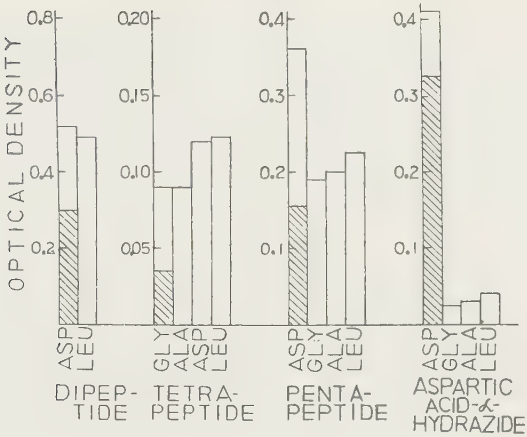
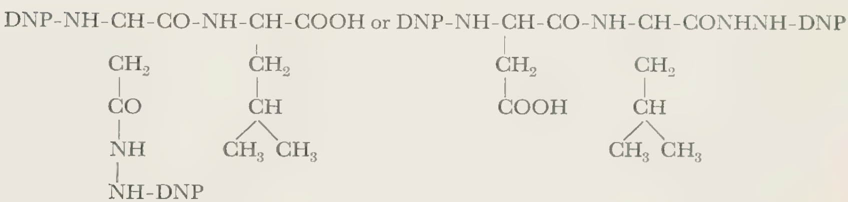


FIG. 2. Amino acid compositions of DNP-peptides.

//////......DNP-Amino acid obtained by extraction of the hydrolysate.

☐......DNP-Amino acid obtained by dinitrophenylating the residual amino acids.



(derived from C-terminal asparaginyl-leucine)

(derived from aspartyl-leucyl-residue in inner peptide)

However, in view of the results cited in Table II and the analytical data of C-terminal peptide mixture, it is highly probable that this peptide derivative is derived from C-terminal asparaginyl-leucine. About the component VI, it is not yet clear whether the β-carboxyl group of the N-terminal aspartic acid is free or amide merely from this result, although it might be possible to determine by hydrazinolysing again the component VI followed by dinitrophenylation, whereupon the residue is identified as aspartyl if di-DNP-aspartic acid-α-hydrazide is detected, and as asparaginyl if the hydrazide is not found.

DISCUSSION

Thompson (*12*) reported that carboxypeptidase released only C-terminal leucine from lysozyme with reaction period of up to 24 hours, and after that time several amino acids were liberated in small quantity almost simultaneously, although she had originally hoped that, by following the liberation of other amino acids during the reaction, the sequence of number of residues along the chain from the leucine end-group could be revealed. This fact might be explained from the C-terminal peptide sequence and the specificity of the carboxypeptidase.

The specificity of acrboxypeptidase is known to be concerned with the nature of the residues of two subsequent amino acids. Although no data on the rate of hydrolysis of alanyl-asparaginyl-leucine is available, it may be probable that the rate of spliting of the bond asparaginyl-leucine is far faster than that of alanyl-asparagine. According to Harris (*13*) the rate of release of C-terminal alanine is about 8 times that of the asparagine in the case of insulin, and alanine itself, too, is said to be far less splittable than leucine by the enzyme—rate of about one-hundredth, roughly (*14*)—so that after releasing the C-terminal leucine from lysozyme, it might be very slow to split the next asparagine by the enzyme, and thus the result of Thompson might not be inconsistent with that of the author.

Thompson (*15*) also reported several peptides obtained from partial hydrolysate of lysozyme including 8 sorts of leucine residues. As lysozyme contains 8 lecuine residues per molecule, the C-terminal peptide must be involved among the peptides obtained by her on which the aspartyl-leucine must be the very one.

Comparing the procedure described above to that of the determination of N-terminal peptide (*6*), the following differences may be pointed out:

(i) The DNP-peptides obtained in the case of C-terminal are easily examined in respect of their homogeneity by hydrolysing and chromatographing their N-terminal amino acids. If only one N-terminal DNP-amino acid is detected from each of the DNP-peptides, the peptide is proved to be homogeneous, but if two or more DNP-amino acids appeared, it is regarded as inhomogeneous;

(ii) Acidic amino acid residues in C-terminal peptides are able to be confirmed briefly their mode of linkage;

(iii) As inconvenient to this procedure, acidic amino acids bearing one free carboxyl group and probably their peptide too (both non-C-

terminal), would contaminate the fraction of C-terminal peptides.

Schroeder (*10*) reported four amino acid sequences of lysozyme from N-terminal, so that it is now elucidated that lysozyme is an one-chain polypeptide having the N- and C-terminal sequences as follows: Lys-Val-Phe-Gly-·······-Asp(NH_2) (or Asp)-Gly-Ala-Asp(NH_2)-Leu. The comparative abundance of serine and glutamic acid in the C-terminal peptide mixture suggests the presence of these amino acids near C-terminal.

SUMMARY

1. A new procedure for determining the amino acid sequences from carboxyl-terminal of proteins by means of hydrazinolysis was described.

2. Asparaginyl(or aspartyl)-glycyl-alanyl-asparaginyl-leucine was proposed as carboxyl terminal peptide of lysozyme.

The author wishes to express his deep thanks to Prof. Shiro Akabori for his interest and encouragements throughout this investigation.

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THE ROLE OF NUCLEIC ACIDS AND THE STRUCTURE OF NUCLEOPROTEIN

I. ON THE THIOL GROUPS OF THE NUCLEOPROTEINS OF RABBIT LIVER

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(Received for publication, June 14, 1955)

In recent years much attention has been focussed on nucleic acids as controlling factors in protein synthesis. This is owing to the existence of evidence from which may be deduced certain correlations between chemical findings on the one hand and observation of its biological function on the other. Thus, for example, it is supposed that genes control ultimately all cellular functions, including protein synthesis. From the close association of genes with nucleoproteins it is natural to assume that nucleic acids may be involved in protein synthesis.

On the other hand, it has already been reported that thiol groups play an important role in cell division and cell growth (*1*), but the biological function of SH-compounds has not been completely clarified as yet. The activities of many SH-enzymes are undoubtedly dependent upon their tiol groups (*2*), and it is also well-known that some proteins bind with their prosthetic groups through their thiol groups, through which the proteins may serve their biological functions (*3*). Some of thiol groups in native proteins are " freely reacting " groups which react readily with nitroprusside reagent and mild oxidizing agents such as ferricyanide, iodosobenzoate, and porphyrindine without being denatured.

In 1940, Greenstein *et al.* (*4*) reported that the liver nucleoprotein of rabbit chiefly consisted of desoxyribonucleoprotein (DN-protein) and that almost all of their thiol groups were " freely reacting." If this is true, it may not be denied that some proteins take activated structures through binding with nucleic acids. Moreover, we are deeply interested in the finding of Rapkine (*5*) that the thiol groups of fertilized sea urchin eggs change suddenly from sluggish to freely reacting just before cell division. His finding demonstrates a definite relationship

between cell division and thiol groups. The work reported here was undertaken in the hope that more detailed data on the thiol groups of liver nucleoproteins might be obtained.

EXPERIMENTAL AND RESULTS

The nucleoprotein was extracted from rabbit liver by the method of Greenstein *et al.* (*4*) and the content of its thiol groups was determined by the ferricyanide method (*3*) before and after urea denaturation. The bulk of nucleoprotein thus obtained was found to be ribonucleoprotein (RN-protein) contrary to the finding of Greenstein *et al.*, but it was reconfirmed that most of its thiol groups were certainly freely reacting (Table I). The nucleoprotein, however, was not simply RN-protein but also contained a considerable amount of DN-protein as shown in Table I. Then RN- and DN-proteins were extracted separately by the method of Griffin *et al.* (*7*) and the content of their thiol groups was determined. As shown in Tables II and III, the increase of thiol groups in RN-protein after urea denaturation was only 16.6 per cent, while that

TABLE I

Freely Reacting Thiol Groups of Nucleoprotein and Its Protein Moiety before and after Urea Denaturation

No.	Urea denaturation	SH (M/M protein)	Increase of SH-groups by denaturation	Nitro-prusside test	RNA	DNA
		Nucleoprotein				
3	Before	320	43.8 %	+	84%	16%
	After	460				
4	Before	235	29.7		88	13
	After	305				
7	Before	278	7.2		92	6
	After	280				
Mean	Before	278	26.9		88	12
	After	315				
		Protein moiety				
3	Before	193	115.0	−		
	After	415				
4	Before	185	37.0			
	After	255				
7	Before	90	89.1			
	After	170				
Mean	Before	156	79.4			
	After	280				

Nucleic acids were determined by the modified method of Schneider and Schmidt-Thannhauser (*7*).

TABLE II

Freely Reacting Thiol Groups of Ribonucleoprotein before and after Urea Denaturation

No.	Urea denaturation	SH (M/0.1 mg. N)	SH	Nitroprusside test	Millon's test	N/P	RNA
Ribonucleoprotein							
1	Before	0.072	0.40%	+	−	10.4	3.5%
	After	0.082	0.45				
2	Before	0.086	0.52			10.9	12.4
	After	0.100	0.60				
3	Before	0.080	0.47			10.8	10.0
	After	0.086	0.56				
Mean	Before	0.079	0.50			10.7	8.6
	After	0.089	0.54				
Protein moiety							
1	Before	0.073	0.39	+	−	14.3	
	After	0.072	0.39				
2	Before	0.048	0.25			11.5	
	After	0.048	0.25				
3	Before	0.065	0.34			14.9	
	After	0.065	0.34				
Mean	Before	0.062	0.33			13.6	
	After	0.062	0.33				

of DN-protein was 199.9 per cent. This means that the ratios of freely reacting groups to total thiol groups of both nucleoproteins were 85.5 per cent and 35.2 per cent respectively. The protein moieties from both nucleoproteins were then isolated by the method of Mirsky and Pollister (*8*). The protein isolated from RN-protein precipitated at pH 5.3 in HCl solution and was soluble in dilute alkaline solution. Biuret test were positive in both proteins. Millon's test was positive only in the protein isolated from DN-protein. This protein moiety of DN-protein was considered to be a histone-like protein and that of RN-protein to be neutral or acid protein. The ratio of freely reacting thiol groups to total thiol groups was 36.3 per cent in the former and 100 per cent in the latter, as shown in Fig. 1.

DISCUSSION

Spiegelman and Kamen (*9*) reported that ribonucleic acid was extremely important in controlling protein synthesis, and many workers now support this view. The fact that almost all the thiol groups of liver nucleoprotein, especially of RN-protein, are freely reacting may

TABLE III

Freely Reacting Thiol Groups of Desoxyribonucleoprotein before and after Urea Denaturation

No.	Urea denaturation	SH (M/0.1 mg. N)	SH	Nitroprusside test	Millon's test	N/P	RNA
Desoxyribonucleoprotein							
1	Before	0.024	0.17%	−	+	7.8	23.8%
	After	0.081	0.56				
2	Before	0.074	0.56			5.5	30.0
	After	0.166	1.26				
3	Before	0.011	0.08			3.3	29.7
	After	0.038	0.28				
Mean	Before	0.036	0.27			5.5	27.8
	After	0.095	0.70				
Protein moiety							
1	Before	0.051	0.27	−	+	50.5	
	After	0.145	0.76				
2	Before	0.019	0.10			59.3	
	After	0.061	0.32				
3	Before	0.023	0.12			42.3	
	After	0.054	0.28				
Mean	Before	0.031	0.16			50.7	
	After	0.087	0.45				

have some bearing on the fact that thiol group itself plays an important role in cell division and cell growth. In any case, it was reconfirmed that RN-protein had an activated structure, at least through its thiol groups. Although almost all the thiol groups of the protein moiety itself isolated from RN-protein remained freely reacting contrary to our expectations, our assumption that protein may be activated structuarlly through binding with nucleic acids cannot be wholly denied because proteins are apt to suffer denaturation in many steps when being isolated from nucleoproteins.

SUMMARY

1. Majority of the nucleoproteins isolated from rabbit liver by the method of Greenstein *et al.* was RN-protein.

2. Almost all of its thiol groups were freely reacting. The mean ratio of the freely reacting thiol groups to the total thiol groups of RN-

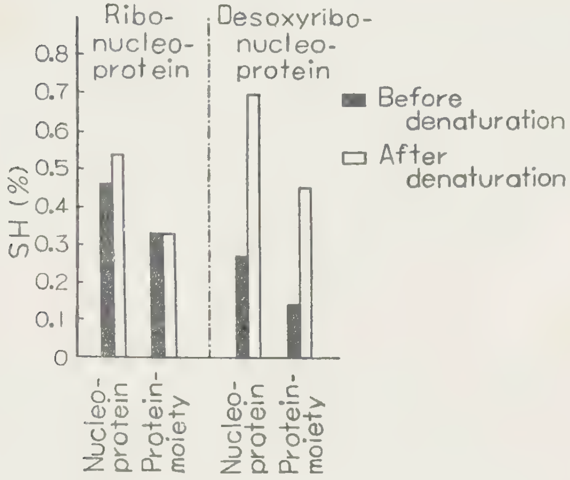

FIG. 1. Freely reacting thiol groups of ribo- and desoxyribo-nucleoprateins and of their protein moieties before and after urea denaturation.

protein was 85.5 per cent, while that to the DN-protein was 34.8 per cent.

3. The protein moiety of DN-protein may be considered to be a histone-like protein and 36.3 per cent of its total thiol groups was freely reacting.

4. The protein moiety of RN-protein may be neutral or acid protein, and all of its thiol groups were freely reacting.

The author wishes to his gratitude to Prof. J. Hirade and Dr. T. Shimizu for their advices and encouragement in this research.

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The Journal of Biochemistry, Vol. 42, No. 6, 1955

THE ROLE OF NUCLEIC ACIDS AND THE STRUCTURE OF NUCLEOPROTEIN

II. ACTIVATION OF PROTEINS BY NUCLEIC ACIDS

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(Received for publication, June 14, 1955)

In the previous paper (*1*) the authors reported that most of the thiol groups of ribonucleoprotein of rabbit liver were freely reacting and those of the protein moiety isolated from ribonucleoprotein were also freely reacting. Whether it was due to the binding with ribonucleic acid (RNA) or merely to the structural changes during the isolation procedure, that the protein moiety of ribonucleoprotein had activated structure as for thiol groups, remained to be clarified, but it is not quite unreasonable to consider that through binding with RNA protein may take an activated structure. To clarify the structural activation of some radicals of the protein binding with RNA the following experiments were undertaken by the present authors.

EXPERIMENTAL

Proteins used in the experiments were ovalbumin (OA), horse serum albumin (SA), lactoglobulin (LG), and β-lactoglobulin (β-LG). OA and SA were prepared by the Kekwick's method (*2*), and LG and β-LG by the Palmer's method (*3*). As RNA, yeast sodium nucleate (Merck) was used.

Free amino acid and thiol groups were measured by the modified formol titration method (*4*) and by the Folin's method (*5*), respectively. The alkylation velocity of free amino groups was measured by determining the decrease of free amino groups in the presence of iodoacetamide (IAm).

RESULTS

Free Amino Groups—OA and RNA (each in 2M NaCl solution) were mixed in various percentages and the free amino groups were determined after incubating for 10 minutes at 25°. As shown in Fig. 1, the free amino groups increased to the extent of 64 per cent of the free amino groups of OA itself when incubated with RNA. It was shown that the effect of RNA on the free amino group increase is more distinct in

0.5M NaCl solution than in 2M NaCl solution. Therefore, all experiments hereafter were carried out in 0.5 M NaCl solution.

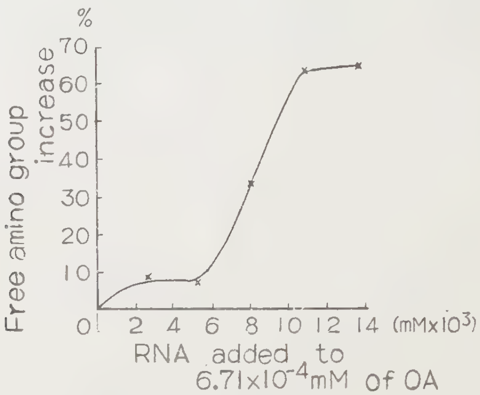


FIG. 1. Increase of amino group of OA by RNA.

The same experiments as above were carried out with SA, LG, and β-LG at 35° and it was found that free amino groups increased markedly in all cases by the addition of RNA (Fig. 2). The relation of the amino group increase to RNA added was not linear but showed a Sigmoid curve. It is noteworthy that in the case of SA, there were three points of inflexion before showing rapid increase of free amino groups, as shown in Fig. 2.

Alkylation Velocity—The alkylation velocity of OA with IAm was measured at 2–3.5°, 25° and 38°. As shown in Fig. 3, OA itself did not react with IAm even at 38°, within at least 3 hours, but when added with RNA, OA reacted with IAm and a noticeable alkylation velocity was observed. The temperature coefficient of this velocity was far less than that of ordinary chemical reactions and was only +13 per cent between 25° and 38°. (Table I).

The results of this experiment suggest that this reaction may be controlled primarily by some physical factors such as the revealing of sluggish amino groups besides pure chemical factors. The raction velocities of other proteins also increased markedly with the addition of RNA. The reaction systems are given in Table II. As for SA, the existence of two kinds of amino groups with different reactivity was assumed from the reaction curve (Fig. 4).

Thiol and Disulfide Groups—OA had no freely reacting thiol groups, but when incubated with RNA at 25° for 15, 30, or 120 min., freely reacting thiol groups appeared markedly as shown in Fig. 5, while freely reacting disulfide groups of SA showed no change through binding with RNA but those of SA previously denatured by urea increased through binding with RNA (Fig. 6). The results of this experiment seem to indicate that the disulfide groups have a more rigid structure than the amino groups

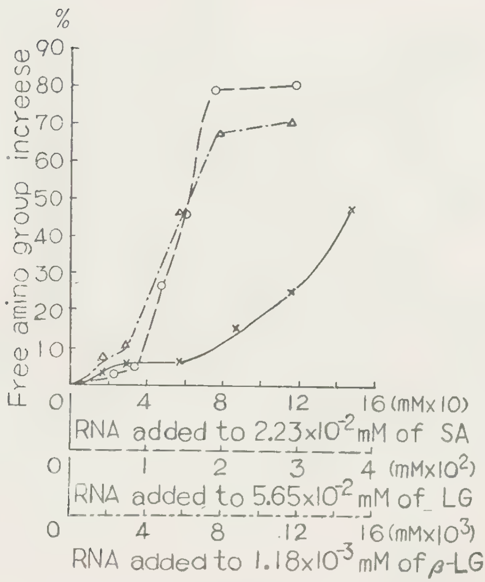


FIG. 2. Increase of free amino group of SA, IG, and β-LG by RNA.
×——× SA ○– – –○ LG △– - – - –△ β-LG

TABLE I

Relation between Temperature and Alkylation Velocity of OA with IAm

Temp. (°C)	Reaction velocity (*mol./min.* $\times 10^2$)
2-3.5°	1.67
25°	3.93
38°	4.45

and that, after partial unfolding of the protein structure by urea denaturation, disulfide groups could be revealed through binding with RNA.

Viscosity—The viscosities of OA and SA were measured by the Ostwald's method. As shown in Table III, they decreased markedly with the addition of RNA and this indicates that there is apparently an interaction between protein and added RNA.

Activation of Urease—If our assumption that protein may take an activated structure when RNA is added to a protein is correct, the activities of some enzymes may be increased through binding with RNA. From this point of view, the activity of urease was measured by the Van Slyke's method (*6*) with and without the addition of RNA

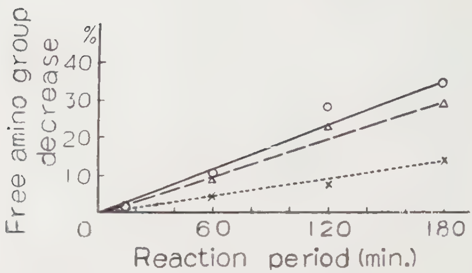


FIG. 3. Effect of RNA on the alkylation velocity of amino groups of OA with IAm.

OA	RNA	Phosph. buffer	IAm	NaCl	pH
6×10^{-2}mM	3.03×10^{-1}mM	1.67 mM	50 mM	6.7×10^{-2} mM	6.8

○—○ 38° △— —△ 25° ×- - - -× 2-3.5°

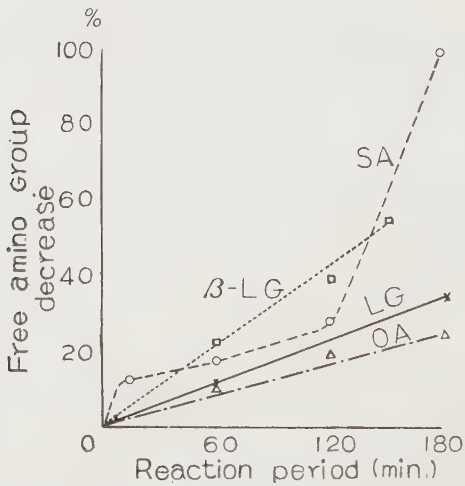


FIG. 4. Effect of RNA on the alkylation velocity of amino groups of proteins with IAm. Temperature at 35°.

and it was observed that the activity of urease increased with the addition of RNA, this increase followed the increase of the free amino groups (Fig. 7), and that the increase of the activity was the maximum at pH 7.5 (Fig. 8).

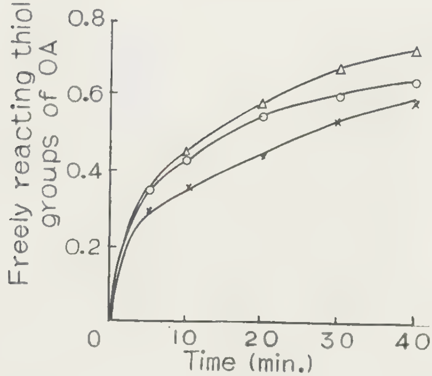


FIG. 5. Activation of thiol groups of OA by addition of RNA.
Ordinate scale——mole/mole Condition : 25° pH : 6.8.

0.865% OA	2% RNA
2.0 ml.	2.0 ml.

△——△ Incubated with RNA for 120 min.
○——○ ,, ,, ,, 30 min.
×——× ,, ,, ,, 15 min.

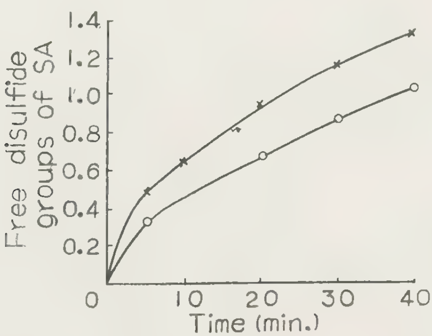


FIG. 6. Activation of disulfide groups of SA by addition of RNA after urea denaturation.

×——× : SA was added with 1 g. of urea, maintained at 28° for 15 min., and then added with RNA.

○——○ : Same as above but without RNA.

1.155% SA	2% RNA	Urea
2.0 ml.	2.0 ml.	1 g.

Ordinate scale—mole/mole.

TABLE II

Reaction System of Alkylation of Protein with IAm Reaction Systems

Protein		RNA	IAm	Buffer	NaCl	Temp.(°C)	pH
	M	M	M	M	M		
OA	6×10^{-5}	3.03×10^{-4}	5×10^{-2}	1.67×10^{-3}*	0.67	38	6.8
SA	7.4×10^{-5}	3.24×10^{-3}	5×10^{-2}	8.33×10^{-2}*	0.5	35	6.8
LG	2.4×10^{-5}	6.26×10^{-6}	9.6×10^{-2}	1.85×10^{-3}**	0.5	35	6.8
β-LG	2.1×10^{-5}	5.11×10^{-5}	4.4×10^{-2}	2.73×10^{-3}**	0.5	35	6.8

* Phosphate Buffer. ** Borate Buffer.

TABLE III

Effect of RNA Addition on the Intrinsic Viscosity of OA and SA

Protein	RNA	Intrinsic viscosity	
		Rod	Disc
OA	−	4.55	5.80
	+	2.90	3.20
SA	−	5.19	5.60
	+	3.20	2.60
−	−	5.05	6.70

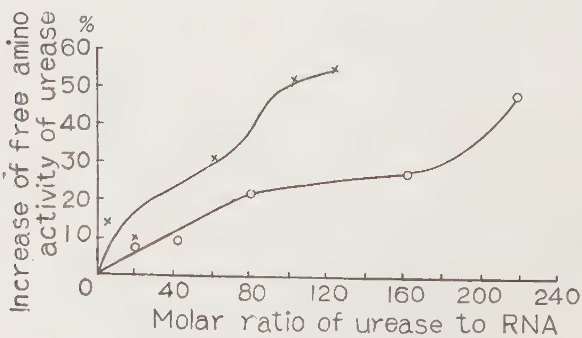


Fig. 7. Effect of RNA on the free amino groups and activity of urease.

0.5 M NaCl ×—× Free amino groups.
35°, 10 min. ○—○ Activity.

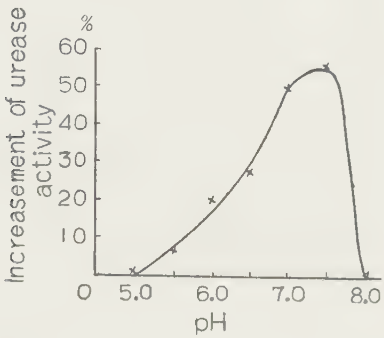


FIG. 8. Relation between pH and increase of urease activity by addition of RNA.

Molar concentration of enzyme system ($\times 10^{-3}$ M)

Urease	RNA	Urea	Phos. buffer	NaCl
2.79×10^{-3}	9.9×10	5.82×10^{-1}	6×10	5×10^{2}

DISCUSSION

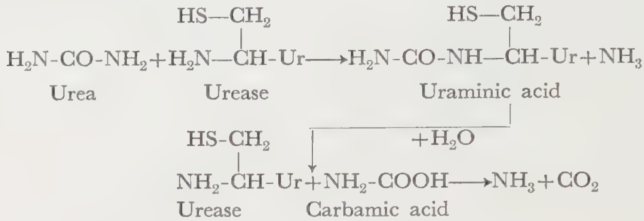
Nucleic acids attract not a little attentions as the templates for the *in vivo* synthesis of protein, and its phosphate bonds are regarded as serving some special functions for various reactions *in vivo.* However, there have been a few reports concerning the activation of the protein structure through binding with nucleic acid.

Our present data show that some groups of proteins may be unfolded through binding with RNA and if these groups play an important role in biological functions of proteins, biological activity of protein must be intensified through binding with RNA and our results may suggests one role of RNA in the cellular structure.

The mechanism of intensifying urease activity by RNA may consist of unfolding the amino groups of urease protein by the addition of RNA, through which uraminic acid may be formed with urea, as shown in the following schema, and as the result the activity of urease may be intensified.

From the fact that both relative viscosity and flow birefringence of desoxyribonucleic acid decrease remarkably when added with OA or SA, Greenstein (7) pointed out the possibility that asymmetric structure of desoxyribonucleic acid is changed by the addition of these pro-

teins. However, the present series of experiments induces us to emphasize the possibility that the protein itself may be structurally activated through binding with nucleic acid.



SUMMARY

1. Free amino and thiol groups of various proteins increased when incubated with ribonucleic acid.

2. Ribonucleic acid increases free disulfide groups of serum albumin previously denatured by urea but not native serum albumin.

3. Alkylation velocity of the amino groups of various proteins with iodoacetaminde is accelerated remarkably by the addition of ribonucleic acid.

4. Ribonucleic acid decreases the viscosity of serum albumin and ovalbumin. This shows that there is an interaction between these proteins and ribonucleic acid.

The authors express their deep gratitude to Prof. J. Hirade for his advice and kind guidance.

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ON THE SPECIES DIFFERENCE OF N-TERMINAL AMINO ACID SEQUENCE IN HEMOGLOBIN. I

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Species specificity or difference of proteins, having similar functions, physical properties, and chemical composition, has been postulated to be due to the difference of the amino acid sequences in their polypeptide chains, without any appreciable direct evidence. Recently, however, Akabori (*1*) suggested that the B-chain of insulin from fish had -Pro-Lys(COOH)* as the C-terminal sequence, differing from that of -Pro-Lys-Ala(COOH) from bovine (*2*). According to Thompson (*3*), human serum albumin has (NH_2)Asp-Ala- as the N-terminal sequence, though it is (NH_2)Asp-Thr- in bovine. Thus, the species difference seems to involve the removal or replacement of some amino acid residues in the corresponding peptide chanis.

On the other hand, Porter and Sanger (*4*) reported that equine hemoglobin molecule has six valyl peptide chains, including (NH_2)Val-Leu- and (NH_2)Val-GluNH_2-Leu (*6*), whereas human hemoglobin five valyl chains and bovine, goat, and sheep ones two valyl and two methionyl chains. So the difference appears to be rather complicated, in spite of their similarity in the composition, molecular weight, and the function. However, the difference in the number of peptide chains may be simple, such as the combination of two chains into one chain. And two terminal methionine in ruminant hemoglobin may correspond to the terminal valine either in (NH_2)Val-Leu- or (NH_2)Val-Glu- of the equine one. These facts prompted us to study the chemical structure of hemoglobin from various animals in more detail.

In the present work, the N-terminal sequence of hemoglobin from dog, horse, pig, bovine, sheep, goat, rabbit, guinea pig, hen, and snake, were determined by the 2,4-dinitrophenyl (DNP) method of Sanger

* The abbreviation of Brand (*5*) are used to present the name of amino acid residues.

(*4*) and the phenylthiohydantoin (PTH) method of Edman (*7*). The result indicated that the species difference involved the variation in the number of peptide chains, one having three (A,B,C) pairs of peptides and the other two (A,B) and involved the replacement of some amino acid residues in B or C chains or in both of them.

EXPERIMENTS AND RESULTS

Materials—Hemoglobin was recrystallized two or three times from the aqueous solution either as oxygenated or carbonylated one with the addition of ethanol, the optimum concentration of which is shown in the column A of Table I.

TABLE I

Hemoglobin from	a		b	c	d
	O_2-Hb	CO-Hb			
Dog	5(e	30	Val	Val-Leu, Val-Asp	Leu, Asp, Gly
Horse	5(e	25	Val	Val-Leu, Val-Glu, Val-(Leu, Glu)	Leu, Glu, Gly
Pig	20	30	Val	Val-Leu, Val-Glu, Val-(Leu, Glu)	Leu, Glu, Gly
Bovine	30	30	Val, Met	Val-Leu	Leu, Gly
Goat	30	30	Val, Met	Val-Leu	Leu, Gly
Sheep	30	30	Val, Met	Val-Leu	Leu, Gly
Rabbit	25	40	Val	Val-Leu	Leu, Gly
Guinea pig	10	30	Val	—	Leu, Asp, Ser
Hen	10	—	Val	Val-Leu	—
Snake	15	—	Val	—	Leu, Gly

a) Concentration of ethanol in *w./w.* per cent for the cyrstallisation.

b) DNP-amino acids found in the complete hydrolyzate of DNP-globin.

c) DNP-peptides isolated from the partial hydrolyzate of DNP-globin.

d) Amino acids adjacent to the N-terminal.

e) Recrystallization was carried out on the dialysate of the solution in alkaline buffer (pH 8.0).

N-Terminal Amino Acids—The usual DNP-method (*4*) was used. DNP-amino acids were extracted three times with ether from the complete hydrolyzate of DNP-globin (100 mg., 6*N* HCl 10 ml., 120–125°, 12 hours), chromatographed on buffered Hyflo-

super-Cel column with ether or chloroform (*8*), and identified by the reproducible R values, shown in Table II. In the remaining aqueous layer of the above extraction,

TABLE II

R-Values of DNP-Amino Acids on Buffered Hyflosuper-cel Column

Mobile phase	Ether			Chloroform			
pH of stationary phase	5.2	6.5	7.4	5.2	6.0	6.5	7.4
DNP-Aspartic acid	0.1	slow	slow	—	—	slow	slow
DNP-Serine	0.3	0.02	slow	—	—	slow	slow
DNP-Glutamic acid	0.4	0.04	slow	—	—	slow	slow
DNP-Threonine	0.6	0.07	slow	—	—	slow	slow
DNP-Glycine	1.0	0.1	slow	—	—	slow	slow
LNP-Alanine	fast	0.16	0.1	—	—	slow	slow
DNP-Methionine sulfoxide	slow	slow	slow	slow	slow	slow	slow
DNP-methionine	fast	0.45	0.2	0.7	0.4	0.1	slow
DNP-Valine	fast	0.7	0.3	0.75	0.5	0.1	0.07
DNP-Phenylalanine	fast	0.75	0.4	0.9	0.6	0.15	0.1
DNP-Leucine	fast	0.8	0.55	fast	0.7	0.2	0.15
2,4-Dinitrophenol	fast	0.75	0.35	fast	0.7	0.5	slow
2,4-Dinitroaniline	fast	fast	fast	fast	fast	fast	fast

no yellow N-DNP-amino acids, except ε-mono-DNP-Lys, could be detected by paper chromatography with solvent A, B, and C of Biserte and Osteux (*9*).

The identification of DNP-α-amino acids derived from N-terminal amino acids was further verified by the method of Lowther (*10*). A part of the ether-soluble DNP-amino acids was treated with ammonia ($d.=0.88$) in sealed tube at 100° for two hours to remove the 2,4-dinitrophenyl radical and the regenerated free amino acids were identified by paper chromatography with phenol-water (4:1), collidine-water (2:1), and butanol-acetic acid-water (4:1:1). All results agreed completely.

As shown in the column B of Table I, valine was the sole N-terminal amino acid in all of the hemoglobin (horse, pig, dog, rabbit, guinea pig, hen, and snake), except those of ruminants (bovine, goat, and sheep), the N-terminal of which was valine and methionine.

N-Terminal Sequence—DNP-globin (100 mg.) was treated with 5 ml. of 12 N HCl at 100° for one hour, diluted to 20 ml. with water, then extracted five times with ether. The ether-soluble DNP-peptides were chromatographed on multi-column with chloroform as the developer. Relative position of each band is summarised in Fig. 1.

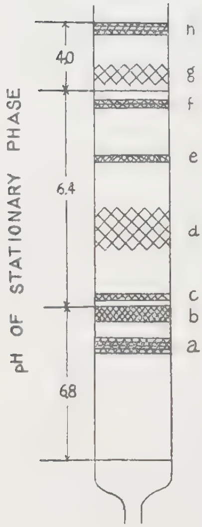
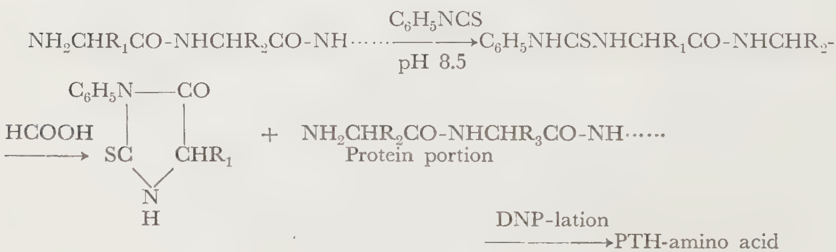


FIG. 1. Chromatogram of the partial hydrolyzate of DNP-globin.

Each DNP-peptide was eluted from the yellow band with four per cent sodium bicarbonate, reextracted with ether from the acidified eluate, then rechromatographed. The structure of purified DNP-peptide was examined by the identification of DNP- and free amino acids in the complete hydrolyzate (6 *N* HCl, reflux for 6 hours), using the above mentioned column- and paper-chromatography, respectively. The results indicated the existence of a) DNP-Val-Leu, b) DNP-Val, c) DNP-Met, d) mixture of DNP-valyl peptides, e) DNP-Val-Glu-Leu or DNP-Val-Leu-Glu, f) DNP-Val-Glu or DNP-Val-Asp, g) mixture of DNP-Val peptides and h) mixture of ε-mono-DNP-Lys and its peptides.

In the case of bovine, goat, and sheep, majority of the terminal methionine was found as free DNP-Met and the amount of free DNP-Val was far less than in the other cases. DNP-Val-Glu could be isolated only in the case of horse and pig, and DNP-Val-Asp in dog. On the other hand, almost the same amount of DNP-Val-Leu could be found throughout all kinds of hemoglobin (bovine, goat, sheep, horse, pig, rabbit, dog, and hen). These results are shown in the column C of Table I.

Amino Acid Residues Adjacent to N-Terminal—Thompson's method (*3*), a combination of Edman's PTH (*7*) and Sanger's DNP (*4*) method, had been used successfully, to determine the amino acid residues adjacent to the N-terminal of hemoglobin molecule.



To five per cent aqueous solution of hemoglobin (100 mg.), five per cent solution of phenylisothiocyanate (100 mg.) in dioxane was added and the brown mixture was agitated at room temperature for six hours. The pH was occasionally adjusted at 8.5 ± 0.5 with 0.01 *N* sodium hydroxide during the whole period. Then, 20 ml. of ethanol was added to the mixture to complete the precipitation of swollen phenylthiocarbamyl (PTC) globin. The PTC-globin (about 100 mg.) after washing well with ethanol and ether, was treated with 2 ml. of 90 per cent formic acid at room tem-

perature for 1.5 hours, to cyclize and split the terminal PTC-amino acid residues as phenyl-thiohydantoin (PTH). To the swollen reaction mixture was then added 20 ml. of ethyl acetate and the precipitated protein portion was separated by centrifugation. The N-terminal amino acids of the well-washed protein portion were determined by the above mentioned DNP-method.

In all cases, two (leucine, glycine) or three (leucine, glycine or serine, glutamic or aspartic acid) kinds of amino acids were found in similar amount together with less amount of valine (valine and methionine in the case of bovine, goat, and sheep), and a negligible amount of serine. Under similar conditions, ovalbumin gave only a trace of serine. The main amino acids detected by this method are shown in the column D of Table I.

DISCUSSION

From the experimental results described above, it might be concluded that hemoglobin from various animals have the following N-terminal sequences:

	(A)	(B)	(C)
Horse, pig	Val-Leu-	Val-Gly-	Val-Glu-
Dog	Val-Leu-	Val-Gly-	Val-Asp-
Vovine, goat, sheep	Val-Leu-	Met-Gly-	—
Guinea pig	Val-Leu-	Val-Ser-	Val-Asp-
Rabbit, snake	Val-Leu-	Val-Gly-	—
Hen	Val-Leu-	?	?

It has been already elucidated that equine hemoglobin molecule consists of six valyl peptide chains (*4*), including Val-GluNH$_2$-Leu- and Val-Leu- sequences (*6*), by Sanger with DNP-method. The present found authors the third sequence Val-Gly- together with the above two, as DNP-Val-Glu, DNP-Val-Leu, and DNP-Val-(Leu, Glu) were found in the partial hydrolyzate of the DNP-globin, and leucine, glycine, and glutamic acid were found as the amino acid residues adjacent to the N-terminal ones by Thompson's method. The terminal amino acid, valine was also found but in less amount, indicating the incompleteness of the thiocarbamylation of hemoglobin. Considering the results of Thompson on serum albumin (*3*) and of the present author on ovalbumin having no N-terminal (*11*), however, leucine, glycine, and glutamic acid in equine hemoglobin seemed to be the true amino acids adjacent to the N-terminal, and not to be the artificial one, such as serine, which was liberated in negligible amount by acyl-migration (*12*) during the formic acid-treatment. As these three amino acids were found in equal amount and equine hemoglobin

molecule had six valyl chains (*4*), it must be consisted from a pair of three different peptide chains, as (A:Val-Leu-)$_2$ (B:Val-Gly-)$_2$ (C:Val-GluNH$_2$-Leu-)$_2$ and may dissociate into one-half A_1 B_1C_1 by the urea-denaturation (*13*). Perutz *et al.* (*14*) suggested a model of equine hemoglobin which consisted of three pairs of different peptide chains in their length, from the results of their X-ray analysis. The studies on the possible relationship between the three different chains proposed from the physical data and from the N-terminal amino acid sequence are in progress.

Quite similar results were obtained on pig hemoglobin and the species difference between horse and pig could not be proved as far as examined. On the other hand, dog hemoglobin had Val-Asp- sequence instead of Val-Glu- in the C-chain of equine one, indicating the replacement of similar amino acid residues. According to Sanger (*6*), equine hemoglobin had Val-GluNH$_2$-Leu- sequence, so it may be Val-AspNH$_2$- in dog hemoglobin.

In bovine, goat, and sheep hemoglobin, only two amino acids, leucine and glycine, were found in an equal amount, as the amino acid adjacent to the N-terminal. As these DNP-globin gave two DNP-Val and two DNP-Met on complete hydrolysis (*4*) and gave DNP-Val-Leu on the partial hydrolysis, the following structure may be possible: (A:Val-Leu-)$_2$ (B:Met-Gly)$_2$ or (A:Met-Leu-)$_1$ (A′:Val-Leu-)$_1$ (B:Met-Gly-)$_1$ (B′:Val-Gly-)$_1$. The former structure A_2 B_2, may be strongly suggested from the following facts, that in the partial hydrolyzate of the ruminants DNP-globin, majority of the terminal methionine was found as free DNP-methionine, the amount of free DNP-valine was far less, and the similar amount of DNP-Val-Leu was found as compared with the other cases.

In the cases of rabbit and snake, the structure (A:Val-Leu-)$_m$ (B:Val-Glu-)$_m$ was concluded without question. Hemoglobin from guinea pig had equine-type structure: (A:Val-Leu-)$_n$ (B:Val-Ser-)$_n$ (C:Val-Asp-)$_n$ and the seryl and aspartyl (or aspargyl) residues seemed to correspond to glycyl and glutamyl (or glutaminyl) residues of equine one, respectively, thouoh it is still uncertain that m and n are equal to two.

Some years ago, Sanger (*14*) reported the difference of amino acid composition in the A-chain of insulin from bovine, sheep, and pig, and suggested that the species difference involved the replacement of glycine and alanine with serine and threonine, respectively. The replace-

ment of latter type was verified on serum albumin by Thompson. The present author could elucidate the replacement of former type on hemoglobin from above mentioned animals, together with two new ones between glutamine and asparagine, methionine and valine. The remarkable difference of N-terminal sequence in both rodentia hemoglobin from guinea pig and rabbit, and in both *Artiodactyla* (eventoed ungulated) hemoglobin from ruminants (bovine, goat, and sheep) and non-ruminant (pig), seemed to be very curious. Such a difference in the number of peptide chains and the replacement of amino acid residues, however, might determine the species specificity or difference of hemoglobin molecule.

SUMMARY

N-terminal sequences of hemoglobin molecule from various animals were determined by the DNP- and PTH- methods and the following results were obtained:

Horse, pig	Val-Leu-	Val-Gly-	Val-Glu-(Leu)
Dog	Val-Leu-	Val-Gly-	Val-Asp-
Bovine, goat, sheep	Val-Leu-	Met-Gly-	—
Guinea pig	Val-Leu-	Val-Ser-	Val-Asp-
Rabbit, snake	Val-Leu-	Val-Gly-	—
Hen	Val-Leu-	?	?

We wish to express our gratitude to Prof. Akabori for his interest and encouragement through this work, and to Mr. K. Kusama, Mr. K. Titani, and Mr. H. Okuyama for their technical assistance.

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ON THE MECHANISM OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

III. CHROMATOGRAPHIC STUDIES OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

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(Received for publication, June 20, 1955)

Chromatographic analysis on ion exchange resins has been successfully applied to the purification of several enzyme-proteins, for example lysozyme (*1*, *2*), ribonuclease (*3*, *4*, *5*), cytochrome c (*6*, *7*), and α-chymotrypsinogen (*8*). The author has employed the columns of a sulfonated polystyrene resin Dowex-50 to evaluate the homogeneity of the two crystalline proteins, α-chymotrypsinogen and α-chymotrypsin. It was found that the both emzyme proteins could be chromatographed successfully under the same conditions, and the former was eluted with 0.1 *M* citrate buffer of pH 5.51 and the latter with the same buffer of pH 6.77.

In the previous work (*9*), formation of many components of relatively high molecular weight has been confirmed electrophoretically during the activation process of α-chymotrypsinogen to α-chymotrypsin, and they were supposed to be intermediate proteins or liberated peptides. In the present work α-chymotrypsinogen was activated by the method described in the previous report (*9*), and the intermediate stages of the activation were analysed chromatographically by columns of Dowex-50, and the appearance of two intermediate proteins with proteolytic activity was confirmed.

EXPERIMENTAL

Chromatographies of α-Chymotrypsinogen and α-Chymotrypsin with Columns of Dowex-50.

The experimental procedure employed was similar to that described by Moore and Stein (*10*) for the separation of amino acids.

Materials—Chymotrypsinogen was prepared from fresh beef pancreas by the method of Kunitz *et al.* (*11*). It was crystallized four times with ammonium sulfate,

and then once from aqueous ethanol. 1 mg. of the final crystals contained 0.142 mg. protein nitrogen. α-Chymotrypsin was prepared from crystalline α-chymotrypsinogen according to the method of Kunitz *et al.* (*11*), and crystallized once with ammonium sulfate. One mg. of the crystals contained 0.073 mg. protein nitrogen.

Preparation of Ion Exchange Columns—Na-type Dowex-50 (8 per cent cross linkage) purchased from National Aluminate Corporation, Chicago, Illinois, U.S.A., was treated as follows (*cf.* (*10*)).

The resin (250 g.) is ground in water, and passed through 200 mesh screen and then 325 mesh screen with 3–4 liters of water. A uniform particle (200–325 mesh) is first washed with 4 N HCl on a Büchner funnel with very gentle suction. After washing with 2 to 4 liters of the acid and afterwards twice with distilled water, the resin is washed with 2 N NaOH until the filtrate becomes alkaline. The resulting sodium salt of the resin is suspended in about 3 times its volume of N NaOH and heated over a steam bath for about 3 hours with occasional shaking. The supernatant fluid is decanted and replaced with fresh hot N NaOH. This procedure is performed five times in all. Then the resin is filtered and washed free of alkali and stored as the moist sodium salt.

For the preparation of the column, the resin is washed on a filter with a small amount of sodium citrate buffer, pH 3.75 (*cf.* Table I), and suspended in about 2 volumes of the buffer. The slurry (about 15 ml.) is poured into chromatgraph tube through a funnel, the tip of which is bent to direct the stream against the side of the tube. The resin is allowed to settle down under gravity until no further drop in the height of the surface of the resin occurs. The liquid falls to within about 5 cm. on the surface of the resin, whereupon a further addition is made to bring the volume of the column to 10 ml. A separatory funnel (about 200 ml. capacity) containing citrate buffer pH 4.17 is mounted over the column. After about 50 ml. of the buffer is run through to bufferize the resin, the column is ready for use. The chromatograph tubes used in the present experiments were 25 ml. burettes (0.9 cm. diameter), and the composition of the buffers are given in Table I.

Operation of Columns—About an hour prior to use, the prepared column is mounted on a fraction collector. About 1 mg. of crystalline α-chymotrypsinogen is dissolved in 0.3 ml. of citrate buffer of pH 4.17. The resulting solution is placed on the column, and allowed to drain into the column under gravity by washing with three successive 0.3 ml. of the buffer. Then a separatory funnel containing 100 ml. of citrate buffer pH 4.17 is mounted over the column, and operated at room temperature under gravity. The rate of solvent flow through the column is adjusted by the cock of the burette to about 4 ml. per hour, and the effluent is collected in 1 ml. fractions. Solvent exchanges are made in the manner described by Moore and Stein (*10*). Chromatography of about 1 mg. of crystalline α-chymotrypsin was operated in the same manner as in the case of *a*-chymotrypsinogen, and effluent fractions were analysed as follows.

Analysis of Effluent Fractions—Protein measurement has been generally made by the photometric ninhydrin method (*12*). Lowry *et al.* (*13*) have reported the applicability of the Folin phenol reagent to protein measurement by its great sensibility and the

TABLE I

*Preparation of Sodium Citrate Buffers**

Molality for Na	Final pH	Volumes to be mixed**	
		0.2 *M* citric acid	*M* NaOH
M		*ml.*	*ml.*
0.13	4.17	500	130
0.17	4.60	"	170
0.20	5.11	"	200
0.26	5.51	"	260
0.31	6.77	"	310

* Each buffer is 0.1 *M* for citric acid.

** Each mixture is diluted with distilled water to 1,000 ml.

simplicity of the procedure. The author compared the ninhydrin method with the modified Folin tyrosine method (*13*) on the measurement of α-chymotrypsinogen, and found that the Folin Phenol color value was three times larger than that by ninhydrin. Accordingly, the effluent fractions were analyzed by the method of Lowry *et al.* (*13*), with the modification in preparing the solution of 0.5 per cent $CuSO_4 \cdot 5H_2O$ in 1 per cent sodium tartrate by mixing 1 per cent aqueous cupric sulfate with equal volume of 2 per cent sodium tartrate just before use to prevent precipitation. The proteolytic activities of the effluent fractions were determined as described in the previous paper (*9*), and the chromatographic results of α-chymotrypsinogen and α-chymotrypsin are shown in Fig. 1, a and Fig. 1, b, respectively.

Chromatographic Studies of the Activation Process

Activation of α-Chymotrypsinogen—Crystalline α-chymotrypsinogen (293 mg.) was suspended in 6.3 ml. *M*/2 phosphate buffer (pH 7.6) and dissolved by the addition of one or more drops of 5 *N* sulfuric acid, and sodium hydroxide solution equivalent to the acid was also added. The mixture was diluted with distilled water to 25.2 ml., cooled to 5°, and activated at the same temperature by addition of 0.69 mg. of crystalline trypsin* dissolved in 0.5 ml. of 0.0025 *N* hydrochloric acid. Each 0.1 ml. aliquot was taken out at various time intervals, diluted with distilled water to 200 ml. and their proteolytic activities were determined by the method described in the previous work (*9*). The results are shown in Fig. 2. At the same time the activation mixture

* Crystalline trypsin employed was supplied from the Armour and Company, Chicago, Illinois, U.S.A. ; 1 mg. of the crystals contained 0.093 mg. protein nitrogen, and its specific activity was 0.23 $(T.U.)^{cas.}_{mg.P.N.}$

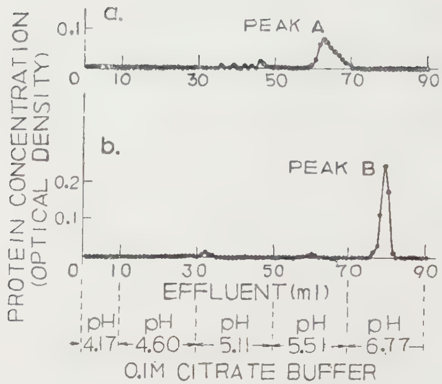


Fig. 1, a. Chromatography of α-chymotrypsinogen.

About 0.5 mg. of crystalline α-chymotrypsinogen was chromatographed. Peak A had no proteolytic activity and could be activated by trypsin as usual.

Fig. 1, b. Chromatography of α-chymotrypsin.

About 1 mg. of crystalline α-chymotrypsin was chromatographed. Proteolytic activity was confirmed only in peak B.

was studied chromatographically with the columns of Dowex-50.

Chromatography of the Activation Mixture—0.3 ml. aliquots taken out at various time intervals were immediately acidified to pH 3.0 with *N* hydrochloric acid, and desiccated in vacuum as fast as possible. The desiccated materials were dissolved in 0.3 ml. citrate buffer of pH 4.17, and chromatographed on columns of Dowex-50 (15 ml. resin) by the method previously described, and the chromatograms of the activation mixture were indicated in Fig. 3.

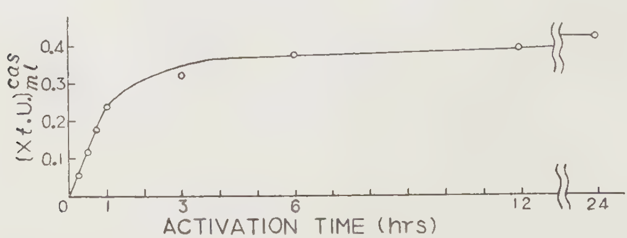
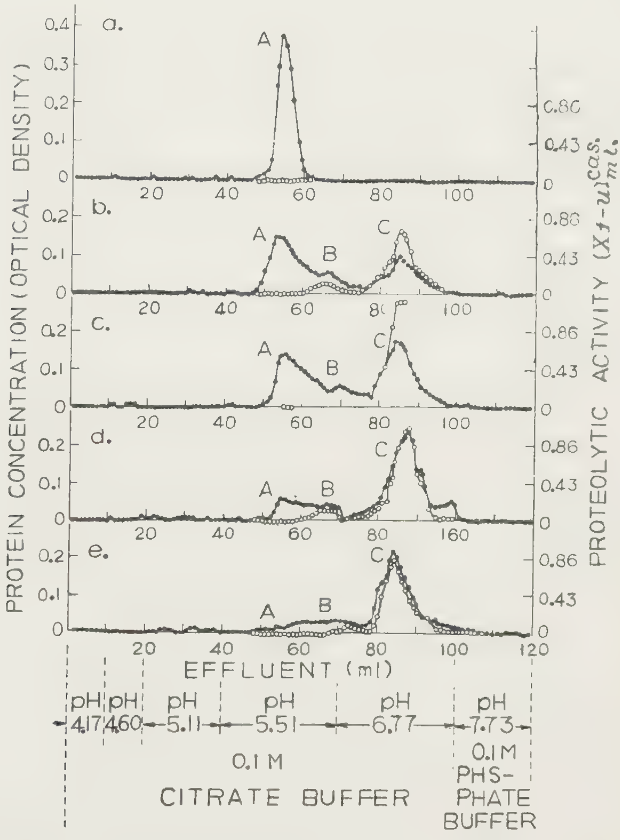


Fig. 2. The Proteolytic Activity during the activation process.

DISCUSSION

The author has previously concluded in accordance with Desnuelle *et al.* (*14*) that α-chymotrypsin had two amino-terminal groups, i.e. alanine and leucine (or isoleucine) (*15*). The other N-terminal



●—● Protein concentration. ○—○ Proteolytic activity.

Fig. 3. Chromatography of the activation mixture.

Fig. 3, 1 is the chromatogram of α-chymotrypsinogen, and Fig. 3, b, 3, c, 3, d, and 3, e are the chromatograms of the activation mixture activated for 0.5, 1, 6, and 24 hours respectively. In each case 0.3 ml. activation mixture (corresponds to 3 mg. of α-chymotrypsinogen) was chromatographed on the columns of Dowex 50 (15 ml. resin).

amino acids detected as dinitrophenyl derivatives were attributed to the contaminated proteins or peptides which were absorbed to or interacted with α-chymotrypsin. In the present paper, it was intended by chromatographies of α-chymotrypsinogen and α-chymotrypsin on columns of Dowex-50 to evaluate their homogeneity, and found that the chromatographic method could be successfully applied to investigate the activation process of the zymogen.

1. It was shown in Fig.1,a that α-chymotrypsinogen could be chromatographed effectively on columns of Dowex-50 with 0.1 *M* sodium cirtate buffer of pH 5.51 (0.26 *M* in Na). A slight asymmetry of the peak A, which was proteolytically active only after the activation by trypsin, might be resulted mainly from the unsuitableness of the pH of the eluent buffer.

2. α-Chymotrypsin could also be chromatographed with 0.1 *M* sodium citrate buffer of pH 6.77 (0.31 *M* in Na) as shown in Fig.1,b, and the material from the peak B was proteolytically active by itself. Therefore, it might be evident that the natures of the two proteins seem not to be affected by chromatography on columns of Dowex-50.

3. Some effluent tubes, except the peak A and B, were shown to be slightly colored by the Folin phenol reagent, but not attended by proteolytic activity. Several attempts were made to prove the existence of another component in these preparations by changing the experimental conditions, but in vain. It is likely to happen that crystalline α-chymotrypsin absorbs tightly small peptides liberated during the activation process, and the peptides give rise to other DNP-amino acids than that of the true *N*-terminal groups of the enzyme protein.

4. The chromatographic result (Fig. 3) shows that α-chymotrypsinogen and its active proteins can be separated to some extent. α-Chymotrypsinogen emerges at 48 ml. effluent as the symmetric peak A, which gradually disappears during the activation process.

5. On the contrary, enzymically active proteins appear only in two inhomogeneous peaks B and C, and the peak C seems to be α-chymotrypsin from its position. However, the peak C might be inhomogeneous and mixed with another highly active intermediate protein, since the ratio of the Folin phenol color value to enzymic activity increases with the activation time. The electrophoretic studies of the activation (*9*) has previously indicated the formation of many components of relatively high molecular weight, some of which may be affected by the chromatographic procedure on columns of Dewex-50 or has the same position on effluent curve as that of the peak B or C.

SUMMARY

1. α-Chymotrypsin could be effectively chromatographed on columns of Dowex-50 with 0.1 *M* sodium citrate buffer of pH 6.77, while α-chymotrypsinogen with the same buffer of pH 5.5. The both proteins seem to be chromatographically homogeous, and not to be affected by the procedure.

2. The activation of α-chymotrypsinogen by trypsin was studied chromatographically. The zymogen and two active proteins were separated by the method, and one of the two active proteins seemed to be α-chymotrypsin accompanied with another highly active intermediate protein.

The author wishes to thank professor Shiro Akabori for his guidance throughout this work, and also Mr. Yoshimi Okada for his valuable technical assistance.

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AUTOÖXIDATION OF L-ASCORBIC ACID AND IMIDAZOLE NUCLEUS

I. THE EFFECTS OF IMIDAZOLE DERIVATIVES ON THE AUTOÖXIDATION OF L-ASCORBIC ACID[1]

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(Received for publication, July 9, 1955)

While the imidazole nucleus is considerably resistant to oxidation by potassium dichromate, nitric acid, or the like, it has been reported that the nucleus of histidine is cleaved to form glutamic acid by so-called histidase system (*2*), though its mechanism of cleavage is as yet obscure. In 1937, S. Edlbacher and A. Segesser (*3*) found that the nucleus of histidine, among other imidazoles, was easily decomposed by aeration in the presence of L-ascorbic acid to liberate ammonia. On the other hand, as it has been reported that the autoöxidation of L-ascorbic acid is affected by the presence of histidine (4, 5), it is highly probable that there are some relations between the autoöxidation of L-ascorbic acid and the decomposition of imidazole nucleus. Despite the lack of sufficient evidences, it has been accepted that L-ascorbic acid may be an intermediate hydrogen carrier of the oxido-reduction systems. However, it has recently become evident that L-ascorbic acid plays a singificant role in the enzyme system of the tyrosine metabolism (*6*). B. B. Brodie and co-workers (*7*) have studied the *in vitro* oxidation of aromatic nucleus in the presence of L-ascorbic acid and found that the reaction products are the same as those formed *in vivo*. These results are of much interest in connection with the biochemical role of L-ascorbic acid.

In this paper the effects of several imidazole compounds on the autoöxidation of L-ascorbic acid and the oxidative degradation of imidazole nucleus are described.

EXPERIMENTAL

Materials

L-*Ascorbic acid*—Commercial preparation from "Wako Pure Chemicals", m.p. 184° (decomp.).

4(5)-Imidazolealdehyde—m.p. 173°. (*8*).

4(5)-Hydroxymethylimidazole—m.p. 92°. (*8*).

4(5)-Imidazolecarboxylic Acid—m.p. 165°. (*8*).

Urocanic acid—m.p. 231° (decomp.). (8).

4(5)-Imidazoleacetic acid-HCl—4(5)-Cyanomethylimidazole (*9*) was hydrolyzed by refluxing with conc. HCl and the evaporated residue was made alkaline with Na_2CO_3 solution. After being boiled to remove NH_3, it was neutralized with HCl, evaporated to dryness and extracted with conc. HCl. The evaporated residue was recrystallized from absolute alcohol. m.p. 227°.

4(5)-Imidazolepropionic Acid—m.p. 204–206°. (*10*).

Imidazole—*4(5)*-Imidazolecarboxylic acid was decarboxylated by heating above 260° and the product was recrystallized from benzene. m.p. 85–87°.

4(5)-Methylimidazole—4(5)-Hydroxymethylimidazole was reduced by refluxing with hydrogen iodide and red phosphorus, the reaction products were saturated with potassium carbonate and extracted with ether continuously. After ether was evaporated, the residue was distilled under reduced pressure. b.p. 163°/20 mm.

Histidine-HCl-H_2O—Commercial preparation from " Ajinomoto Chemicals ", m.p. 250–251° (decomp.).

Histamine-2HCl—Commercial preparation from " Wako Pure Chemicals ", m.p. 228–230° (decomp.).

Methods

Measurements of the Autoöxidation of L-*Ascorbic Acid*—The oxygen-uptake (O_2-uptake) resulting from the autoöxidation of ascorbic acid in the presence of various imidazole derivatives was measured by Warburg's manometer at 37°.

Spectrophotometric Measurements—Ultraviolet spectra were observed by the Beckman type spectrophotometer (Type BQ–50 Shimazu Co.).

RESULTS

Expt. I. The Effects of the Co-existence of Imidazole Derivatives on the Autoöxidation of L-*Ascorbic acid.*

i) *At pH* 7.1 *in Phosphate Buffer*—As shown in Fig. 1, the autoöxidation of ascorbic acid was accelerated both by imidazolecarboxylic acid and by histamine, while imidazolealdehyde, histidine, hydroxymethylimidazole, urocanic acid and methylimidazole inhibited the O_2-uptake (Fig. 1).

Fe^{++} acted against the O_2-uptake of ascorbic acid in the absence of other additions, but somewhat accelerated it in cases where the O_2-uptake had been already diminished by the addition of imidazole derivatives. It had no effect in the presence of imidazolecarboxylic acid (Figs. 1 and 2).

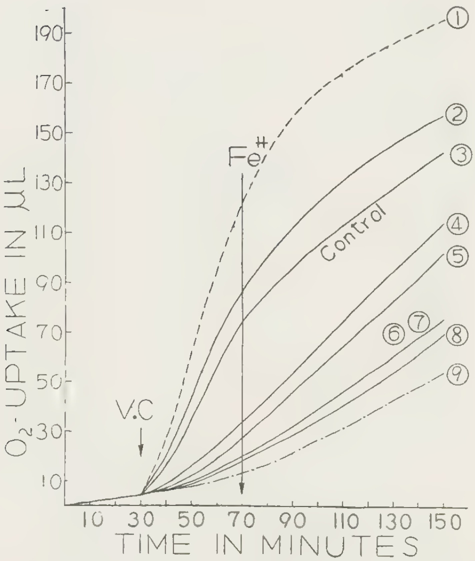

FIG. 1. Effects of several imidazole derivatives and Fe++ at pH 7.1 in phosphate buffer on the autoöxidation of L-ascoric acid.

Basal composition in Warburg's vessels.

Main chamber : Imidazole derivatives 0.25 ml., 5×10^{-6} *M* and other additions. Buffer solution makes the total liquid volume in Warburg's vessel up to 4.0 ml., final concn. 5×10^{-2} *M*.

Side arm 1 : L-Ascorbic acid 0.3 ml., 6.7×10^{-6} *M*.

Side arm 2 : $FeSO_4$ 0.3 ml., 0.81×10^{-6} *M*. (In the following figures, this is not always used.)

Center well : 10% KOH solution 0.2 ml.

Ascorbic acid is added to the main chamber 30 min. after the beginning of the measurements. (In the following figures, these lines are always maintained, except when remarks are made.)

Additions : ① Imidazolecarboxylic acid (- - - -). ② Histamine ③ No addition (L-Ascorbic acid alone)—Control. ④ Imidazolealdehyde. ⑤ Histidine. ⑥ Hydroxymethylimidazole. ⑦ Imidazole. ⑧ Urocanic acid. ⑨ Methylimidazole (•—•—•—).

Fe++ is added to all runs at 70 min.

Imidazolecarboxylic acid rather depressed the O_2-uptake when Cu++ co-existed (Fig. 3).

The degree of inhibition by methylimidazole paralleled the increase

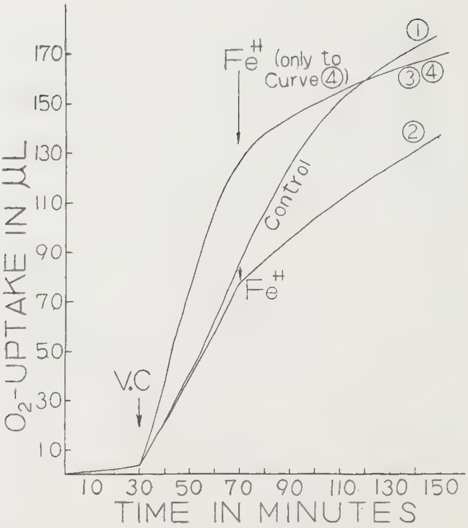

FIG. 2. Effects of imidazolecarboxylic acid and Fe^{++} in phosphate buffer, pH 7.1, on the autoöxidation of L-ascorbic acid.
Additions : ① No addition (L-Ascoric acid alone)—Control.
② Fe^{++} (70 min.) ③ Imidazolecarboxylic acid.
④ Imidazolecarboxylic acid and Fe^{++} (70 min.).

in its concentration and the effect of methylimidazole was overcome by Cu^{++}, the promoting action of the latter being by far greater : thus, in Fig. 4, Curve ③ is depressed to Curve ④ by the addition of methylimidazole (10^{-6} M), but when Cu^{++} (10^{-7} M) is added the curve is raised to Curve ① and the increase in amount of additional methylimidazole to 2.5×10^{-4} M depresses Curve ① to Curve ② again.

Fe^{++} definitely accelerated the O_2-uptake in the presence of methylimidazole ; Curve ⑥ is raised to Curve ⑤ by the addition of Fe^{++} (Fig.4). (The effect was reverse to the case where no imidazole derivative was added (Figs. 1 and 2).

ii) *At pH* 5.8—

a) *At pH* 5.8 *in Phosphate Buffer* (Fig. 5)—In this cases imidazolecarboxylic acid was depressive contrary to the former experiment, and the effect of methylimidazole was similar. (*cf.* Figs. 1 and 2). However, even in the co-existence of Cu^{++}, imidazolecarboxylic acid was as well inhibitory as at pH 7.1 (*cf.* Fig. 3). Fe^{++} also depressed the O_2-uptake definitely.

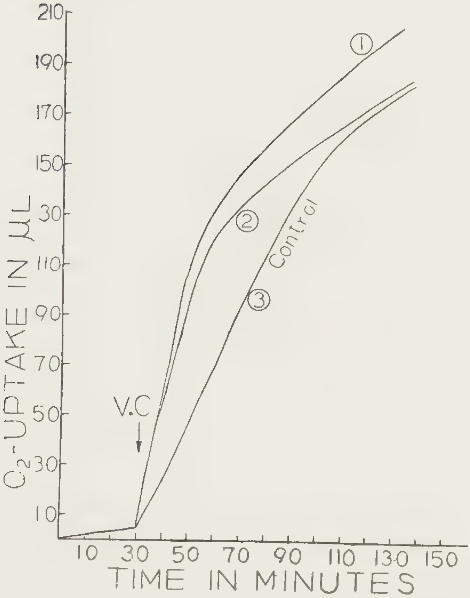

FIG. 3. Effects of imidazolecarboxylic acid and Cu^{++} in phosphate buffer, pH 7.1, on the autoöxidation of L-ascorbic acid.

Additions : ① $CuSO_4$, 10^{-7} *M*, is added to the main chamber at the beginning. ② Cu^{++}, 10^{-7} *M*, and imidazolecarboxylic acid. ③ No addition (L-Ascorbic acid alone)—Control.

b) *At pH 5.8 in Acetate Buffer* (Fig. 6)—Methylimidazole was definitely inhibitory, though its effect was weaker than in phosphate buffer. Fe^{++} affected similarly.

iii) *At pH* 4.3 *in Acetate Buffer*—Imidazolecarboxylic acid definitely depressed the O_2-uptake contrary to the reaction at pH 7.1 in phosphate buffer, and the effect was similar to the case at pH 7.1 and 5.8 in phosphate buffer where Cu^{++} co-existed. The O_2-uptake stopped at about 150 μl. (1 mole per 1 mole ascorbic acid) : it indicates that dehydroascorbic acid, which may be formed in the process, is stable in this conditions (*11*). At pH 7.1 the O_2-uptake exceeded this value (*cf.* Figs. 2, 3 and 4) (Fig. 7).

Contrary to the experiments carried out at other pH values, methylimidazole had almost no effect or somewhat accelerated the O_2-uptake indifferently to the existence of Cu^{++} (Fig. 8). Since the effect of Cu^{++}

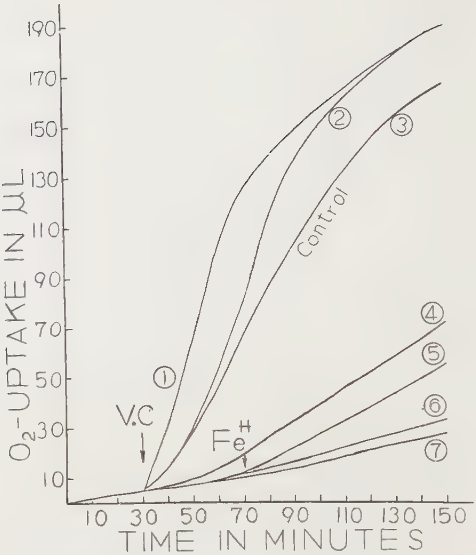

Fig. 4. Effects of methylimidazole, Cu++ and Fe++, in phosphate buffer, pH 7.1, on the autoöxidation of L-ascorbic acid.

Additions : ① Methylimidazole, 10^{-6} *M* and Cu++, 10^{-7} *M*. ② Methylimidazole, 2.5×10^{-4} *M* and Cu++, 10^{-7} *M*. ③ No addition (L-Ascorbic acid alone)—Control. ④ Methylimidazole, 10^{-6} *M*. ⑤ Methylimidazole, 0.5×10^{-5} *M* and Fe++ (70 min.). ⑥ Methylimidazole, 0.5×10^{-5} *M*. ⑦ Methylimidazole, 2.5×10^{-5} *M*.

became negligible at 10^{-9} *M*, it is supposed that the amount of contaminant Cu++ which is present in the reaction mixture is of this order, if one assumes that the O_2-uptake of the control (ascorbic acid alone) is due to the contaminant Cu++ (ascorbic acid hardly absorbs O_2 in metal free re-distilled water (*11*)).

Expt. II *The Ultraviolet Absorption Spectra of the Imidazole Derivatives —L-Ascorbic Acid—Phosphate Buffer System* (*Figs. 9 and 10*).

Since there is no essential difference between Curves ③ and ④, it is concluded that the components co-exist only independently with each other.

DISCUSSION

The rate of the autoöxidation of L-ascorbic acid was affected by

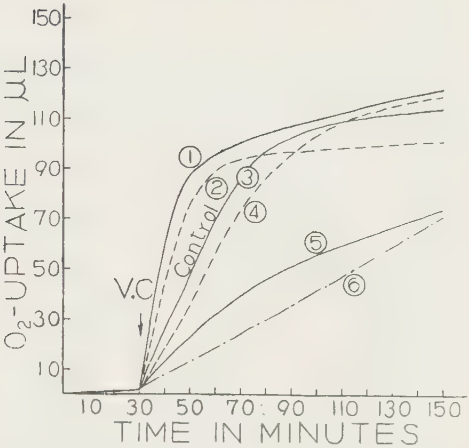

FIG. 5. Effects of imidazole derivatives, Cu++ and Fe++, in phosphate buffer, pH 5.8, on the autoöxidation of L-ascorbic acid.

Additions : ① Cu++, $10^{-7}M$. ② Cu++, $10^{-7}M$ and imidazolecarboxylic acid. ③ No addition (L-Ascorbic acid alone)—Control. ④ Imidazolecarboxylic acid. ⑤ Fe++ (added at the beginning). ⑥ Methylimidazole.

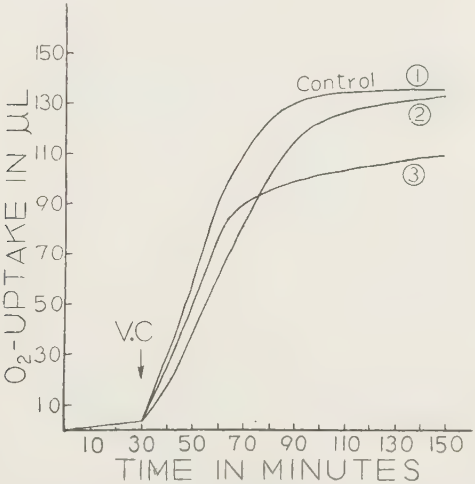

FIG. 6. Effects of methylimidazole and Fe++ in acetate buffer, pH 5.8, on the autoöxidation of L-ascorbic acid.

Additions : ① No addition (L-Ascorbic acid alone)—Control. ② Methylimidazole. ③ Fe++ (added at the beginning).

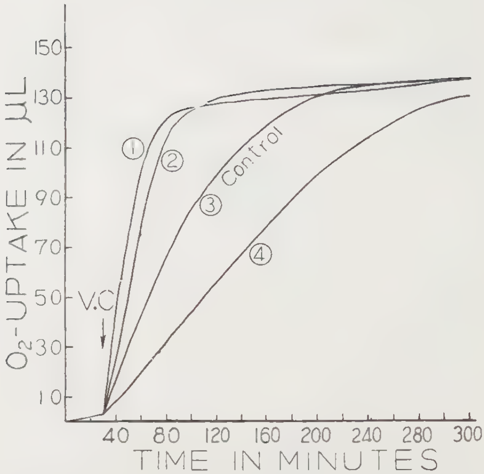

FIG. 7. Effects of imidazolecarboxylic acid and Cu++ in acetate buffer, pH 4.3, on the autoöxidation of L-ascorbic acid.

Additions : ① Cu++, $10^{-7}M$. ② Cu++, $10^{-7}M$, and imidazolecarboxylic acid. ③ No addition (L-Ascorbic acid alone)—Control. ④ Imidazolecarboxylic acid.

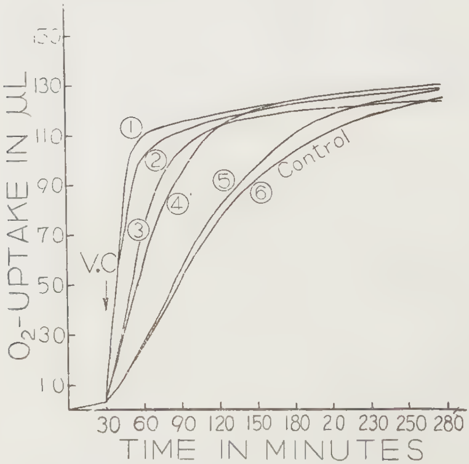

FIG. 8. Effects of methylimidazole and Cu++ in acetate buffer, pH 4.3, on the autoöxidation of L-ascorbic acid.

Additions : ① Cu,++ $10^{-7}M$, and methylimidazole. ② Cu++, $10^{-7}M$. ③ Cu++, $10^{-8}M$, and methylimidazole. ④ Cu++, $10^{-8}M$. ⑤ Methylimidazole. ⑥ No addition (L-Ascorbic acid alone)—Control.

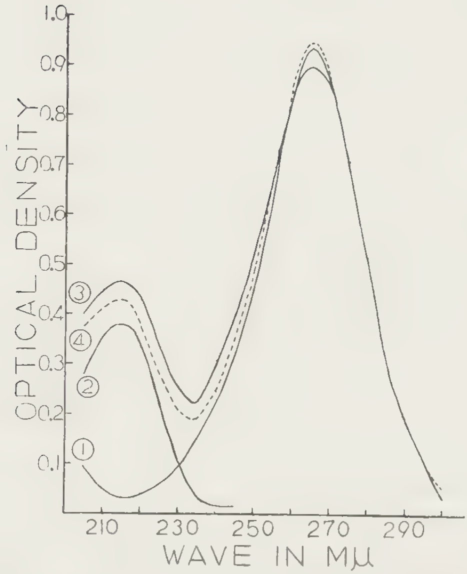

FIG. 9. Ultraviolet absorption spectra of methylimidazole-L-ascorbic acid-phosphate buffer system.

Concentrations of the components are all in both figures $2/3\times10^{-4}M$ in $10^{-2}M$ phosphate buffer (pH 7.1).

① L-Ascorbic acid. ② Methylimidazole (Fig. 9) or imidazolecarboxylic acid (Fig. 10). ③ L-Ascorbic acid and imidazole derivatives. ④ Synthetic curve (①+②) (- - - -).

several imidazole derivatives. As shown in Fig. 1, imidazolecarboxylic acid was the most accelerative, while methylimidazole was the most inhibitory in phosphate buffer at pH 7.1. It is very interesting that the effects of imidazolecarboxylic acid and methylimidazole are reverse to each other in acetate buffer at pH 4.3; namely, that imidazolecarboxylic acid definitely inhibits, while methylimidazole rather accelerates the O_2-uptake (Figs. 7 and 8). At the intermediate pH (pH 5.8) the effects are intermediary. These effects of imidazoles may be attributed to either of the following:

1) The difference in the contaminating Cu^{++} concentration;

2) The difference in the stability of L-ascorbic acid, resulting from some complex formation;

3) The affection to the reaction chain of the autoöxidation of

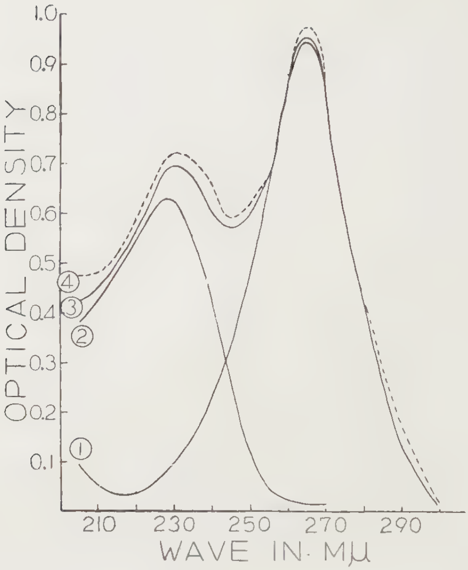

FIG. 10. Ultraviolet absorption spectra of imidazolecarboxylic acid-L-ascorbic acid-phosphate buffer system.
Conditions as in Fig. 9.

L-ascorbic acid.

As for 1), it is conceivable that imidazole derivatives affect the contaminating Cu^{++} concentration, as they usually form stable Cu-salts. That the Cu-salt of imidazolecarboxylic acid is insoluble, while that of methylimidazole is soluble at pH 4.3, does not conflict with the fact that imidazolecarboxylic acid inhibits, while methylimidazole rather accelerates the O_2-uptake. As shown in Fig. 4, the inhibitory action of methylimidazole at pH 7.1 was overcome by the accelerative action of Cu^{++}, it may be of value to take the solubility of the Cu-salts into consideration.

It is interesting that while Cu^{++} definitely accelerates at every pH, Fe^{++} inhibits the oxidation.

As for 2), it is conclusive that any essential difference in the absorption spectra can not be observed among Curve ③ (imidazole derivative—L-ascorbic acid—phosphate buffer system) and Curve ④ which is mathematically synthesized from Curve ① (L-ascorbic acid alone in

phosphate buffer) and Curve ② (imidazole derivative in phosphate buffer). This indicates that no complex formation between L-ascorbic acid and imidazole derivatives may have occurred.

As for 3), the discussion will be made in the following report.

SUMMARY

1. The effects of imidazole derivatives on the autoöxidation of L-ascorbic acid were studied.

At pH 7.1, imidazolecarboxylic acid was the most accelerative, while methylimidazole was the most depressive. These effects reversed at pH 4.3.

2. The ultraviolet absorption spectra of L-ascorbic acid-imidazole derivative—phosphate buffer system was measured, and the possibility of the complex formation between L-ascorbic acid and imidazole derivatives was examined.

3. The effects of these imidazole derivatives were also discussed as regards the differences in the contaminating Cu^{++} concentration.

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AUTOÖXIDATION OF L-ASCORBIC ACID AND IMIDAZOLE NUCLEUS

II. THE DECOMPOSITION PRODUCTS OF IMIDAZOLE DERIVATIVES PRESENT IN THE AUTOOXIDATION MIXTURE[1]

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(Received for publication, July 9, 1955)

In the preceding paper (*2*), the author reported the effects of imidazole derivatives on the autoöxidation of L-ascorbic acid and discussed the results on the following two points:

1) Effects of the contaminating Cu^{++} concentration;

2) Effects of the stability of L-ascorbic acid which may form complex compound with imidazole derivatives.

As mentioned there, histidine, one of the most important imidazole derivatives, and the related compounds are known to be easily decomposed by the aeration in the presence of L-ascorbic acid (*3*). In this paper the results obtained from the studies on the decomposition products of several imidazole derivatives are described and the chain mechanism of the reaction is discussed.

EXPERIMENTAL

Materials

The same as in the preceding paper.

Standard Methods

The Composition of Reaction Mixture—L-Ascorbic acid (1/10 *M* solution) 4 ml.; imidazole derivatives (1/10 *M* solution) 2 ml.; $Fe_2(SO_4)_3$ (1/100 *M* solution) 0.5 ml.; phosphate buffer (1/15 *M* solution at pH 7.1) 3.5 ml. Total volume, 10 ml. After the pH is adjusted to 7.0, the mixture is bubbled moderately with air for 24 hours at 37°.

Detections of the Reaction Products—

i) *Determination of NH_3*—To 2 ml. of the reaction mixture is added 3 ml. of saturated K_2CO_3 solution and steam-distilled for 10 minutes. The NH_3 is absorbed

in 10 ml. of 1/100 N H_2SO_4 and the residual H_2SO_4 is titrated with 1/100 N NaOH using methyl red as the indicator.

ii) *Detection of the Imidazole Derivatives*—After concentration of the reaction mixture in a desiccator, imidazoles are paper-chromatographed using a butanol-acetic acid-water mixture (4:1:1) as the moving solvent. The spots are developed by moistening the paper with Pauly reagent* prepared just before use.

iii) *Detection of the Amino Acids Formed from the Imidazole Derivetives*—After hydrolysis of the reaction mixture by heating at 100° with 3 N HCl for about 3 hours, the concentrated residue is chromatographed on papers using a butanol-acetic acid-water mixtnre (4:1:1) and a phenol-water mixture (4:1) as the moving phases. The spots are developed by 0.2 per cent ninhydrin solution in n-butanol saturated with water.

RESULTS

Expt. I. Decomposition Products of Imidazole Derivatives by Aeration in the Presence of L-*Ascorbic acid*

i) NH_3—The results obtained with several imidazole derivatives are summarized in Table I.**

ii) *Imidazole Derivatives*—No spot other than the starting imidazoles was observed. Rf values are as follows:

Histidine, 0.10 (orange).

Histamine, 0.13 (orange).

Imidazolecarboxylic acid, 0.21 (yellow).

Hydroxymethylimidazole, 0.43 (orange).,

Methylimidazole, 0.55 (red),

Urocanic acid, 0.58 (red),

Imidazolepropionic acid, 0.49 (red).

iii) *Amino Acids*—The results of paper-chromatographies are summarized in Table II.

Expt. II. The Degradation of Hydroxymethylimidazole and Histidine with H_2O_2

---

* A-solution ; 0.9 g. of sulfanilic acid is dissolved in 11 ml. of conc. HCl and diluted to 100 ml. with water.

B-Solution ; 5 g. of $NaNO_2$ is dissolved in water and diluted to 100 ml.

C-solution ; 10% Na_2CO_3 solution.

Each 1 ml. of Solution A and B are mixed, and after about a minute 2 ml. of C are added.

** No NH_3 is formed from hydroxymethylimidazole and histidine by steam-distillation with K_2CO_3 immediately after the reaction mixture was made up. At the end of the reaction with methylimidazole which highly depresses the oxidation of L-ascorbic acid, no ascorbic acid was detected. Then, it is conceivable that L-ascorbic acid may completely be consumed by the aeration when the reaction time is sufficiently long.

TABLE I

NH_3 Formation from Imidazole Derivatives

Imidazole derivatives	Formation of NH_3* (%)		
	mole 1	*moles* 2	*moles* 3
Histidine	94%	47%	31%
Histamine	54	27	18
Imidazolecarboxylic acid	58	29	
Methylimidazole	40	20	
Imidazolepropionic acid	32	16	
Hydroxymethylimidazole	50	25	
Urocanic acid	30	15	

* The values are in terms of % yield, calculated on the assumption that 1, 2, or 3 moles of NH_3 are formed from 1 mole of imidazole derivatives, respectively.

i) *Hydroxymethylimidazole*—

a) *With Aeration*—The L-ascorbic acid solution in the reaction mixture for standard method was replaced by 4 ml. of 1/10 *M* H_2O_2 solution.

Formation of NH_3 5% (2 moles.), 10% (1 mole.)

b) *Without Aeration*—The composition of reaction mixture was as follows:

Hydroxymethylimidazole, 100 mg. in 10 ml., at pH 5-6 (adjusted with HCl), $FeSO_4 \cdot 7H_2O$, 6 mg. in 0.2 ml., and H_2O_2, 44 mg. in 14 ml. of water. The components were mixed (pH 6.3) in an ice-bath and left at room temperature. The control did not contain any hydroxymethylimidazole.

After 3 days:

	Principal	Control
Decomposition of H_2O_2	69%	97%
Formation of NH_3	3.5% (2 moles), 7.0% (1 mole.)	

Table II

Amino Acids Formed from Imidazole Derivatives

Imidazole derivatives	Amino acids formed	Moving solvents			
		Phenol-water		Butanol-acetic-acid-water	
		Rf	(Rf)**	Rf	(Rf)**
Imidazolecarboxylic acid	Glycine	0.39	(0.40)	0.18	(0.17)
Histidine	Aspartic acid	0.10	(0.10)	0.16	(0.16)
Imidazolepropionic acid	Glutamic acid	0.24*	(0.20)	0.16*	(0.14)
Urocanic acid	Glutamic acid	0.22*	(0.19)	0.19*	(0.14)
Methylimidazole	Alanine	not distinct	(0.58)	0.27 0.24*	(0.27) (0.22)
Hydroxymethylimidazole	Serine	not distinct	(0.32)	0.17	(0.14)
Histamine	?	0.65†		0.25†	

* Value of the standard substance which was run mixed with the corresponding test sample, showing the same Rf value as that of latter alone. The Rf of the mixed sample differs occasionally from that of the standard substance alone, but it coincide with that of the test sample.

** ( ) indicates the Rf value of the standard substance alone.

† The product from histamine gives very distinct spot, but has not been identified.

ii) *Histidine*—

a) *Degradation*—The composition of reaction mixture was as follows:

1 g. of histidine in 200 ml., 60 mg. of $FeSO_4 \cdot 7H_2O$ in 2 ml. and 600 mg. of H_2O_2 in 210 ml. of water were mixed in an ice-bath and left at room temperature. The control contained no histidine.

Decomposition of H_2O_2

	Principal	Control
Immediately after the mixing	22%	—
After 1 day	96%	55%
After 3 days	98%	83%

Formation of NH_3 (%)	Principal	Control
	51 (3 moles.), 76 (2 moles.)	—
Results of chromatographies (Rf values)		
Imidazoles	0.10, light yellow (Histidine 0.04, red)	
Amino acids (after hydrolysis)		
Butanol-acetic acid-water	0.11 (0.11), violet-red	
Phenol-water	0.13 (0.13), violet-red	

Thus the amino acid formed coincides with aspartic acid.

b) *Quantitative Determination of Aspartic Acid* (*4*)—The reaction mixture was hydrolysed and concentrated to dryness in order to remove HCl as completely as possible, and dissolved in water. The pH was adjusted to 5.0 with NaOH and diluted to 10 ml. with water. An aliquot of 0.5 ml. was pipetted out and mixed with 10 ml. of water, and the analysis was carried out taking with a 0.4 ml. -portion of this solution. Aspartic acid was estimated by use of pig heart muscle transaminase (*4*).

The principle of the analysis is as follows:

$$\text{L-Aspartic acid} + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{enzyme}} \text{L-glutamic acid} + \text{oxalacetic acid},$$

$$\text{Oxalacetic acid} \xrightarrow{CoCl_2} \text{pyruvic acid} + CO_2,$$

$$\text{L-Aspartic acid} + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{enzyme} + CoCl_2} \text{L-glutamic acid} + \text{pyruvic acid} + CO_2.$$

The amounts of aspartic acid :

CO_2 formed: 67 μl. $\quad \frac{67 \times 133}{22400} = 0.398$ mg.

$$0.398 \times \frac{10.5}{0.4} \times \frac{10.0}{0.5} = 209 \text{ mg. (L-form)}$$

Since the amino acid formed is probably DL-form, the above data is a half of the real value.

$$209 \times 2 = 418 \text{ mg. (DL-form)}$$

The yield is about 66 per cent of the theoretical value calculated from histidine-HCl-H_2O.

c) *Isolation of Aspartic Acid*—After hydrolysis the reaction mixture of a) was neutralized with 6 *N* NH_4OH and again acidified to pH 2.0 with HCl, then boiled with 450 mg. of $CuCO_3$, and dried up on a water bath. The residue was treated with 3 ml. of water and filtered. The filtrate was rubbed with glass rod and the precipitate formed was filtered

off and the clear blue filtrate (pH 5.5–6.0) was left at room temperature. The blue crystals formed were collected and recrystallized from 20 ml. H_2O, washed with water, alcohol, and ether, successively and dried. Blue crystals (25 mg.) resembled the authentic specimen of the Cu-salt of aspartic acid (DL-form) under microscope. The crystals were dissolved in water with 1 drop of 6 N H_2SO_4 (light green-blue, pH 2.0) and saturated with H_2S. The filtrate separated from CuS was freed from H_2S by bubbling N_2 and neutralized with $Ba(OH)_2$. The filtrate from $BaSO_4$ was dried up on a water bath and left in a desiccator over $CaCl_2$. When absolute alcohol was added to the residue, white crystals were formed which weighed 10 mg. The crystals were slightly soluble in alcohol and water, and easily soluble in HCl.

Rf values	0.08 (0.08)	Phenol-water
	0.25 (0.25)	Butanol-acetic acid-water

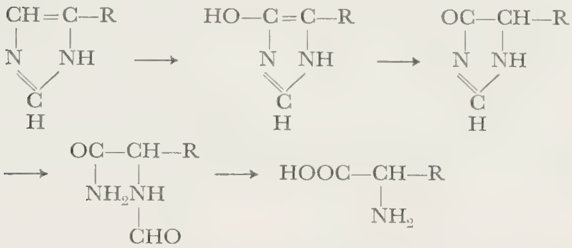
DISCUSSION

It may be assumed that the role of imidazole derivatives in the autoöxidation of L-ascorbic acid is the breaking of the reaction chain of the latter. The greater their reactivity to the intermediate of the reaction chain, the stronger may be their action as chain breaker. The amounts of NH_3 formed may be taken as a measure of their activity as a chain breaker. However, no clear interrelationship was obtained from the results of Experiment I. More NH_3 is formed from histidine than from methylimidazole which is the most inhibitory to the autoöxidation, even if the NH_3 formed from α-NH_2 group is subtracted. If one took not only the nucleus, but also α-amino group into account, histidine should be far more reactive as chain breaker than any other simple imidazoles. But the fact is reverse. It is also contradictory to the above assumption that imidazolecarboxylic acid which accelerates the O_2-uptake produces more NH_3 than methylimidazole as shown in the results. It is, therefore, difficult to clarify the relations between the degree of the O_2-uptake and the NH_3-formation. At any rate, it is conceivable that the degradation of imidazole derivatives in the co-existence of L-ascorbic acid is essentially due to the action of the secondarily formed substance in the autoöxidation of the latter. The most probable intermediate oxidant may be H_2O_2. As it is well known that H_2O_2 is formed in the autoöxidation of L-ascorbic acid (*5*), it is tempting to assume that H_2O_2 formed will attack imidazole nucleus secondarily. However, the

degradation of hydroxymethylimidazole by H_2O_2 is negligible in comparison with the degradation by L-ascorbic acid. Therefore, it is clear that the degradation is not attributable simply to the secondarily formed H_2O_2. Exceptionally histidine reacts as well with 3 moles of H_2O_2 smoothly and is transformed to aspartic acid as with L-ascorbic acid.

If one compares the structures of the amino acids formed and the corresponding starting imidazole derivatives, the site oxidized of the nucleus may be carbon-4(5) as shown in the following Scheme I.

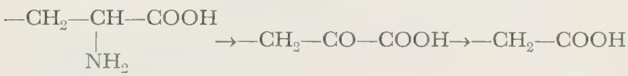
SCHEME I



R is the side chain of various starting imidazole derivatives.

Besides the oxidative rupture of the nucleus, the side chain of histidine may be shortened as follows:

SCHEME II



It was reported that histidine was degraded to aspartic acid by liver tissue (*6*) and by ultraviolet light (*7*). Histamine was converted to aspartic acid by Achromobacter (*8*, *9*). The oxidative metabolic pathway of histidine to aspartic acid may be a general one.

It is very interesting that glutamic acid is formed from urocanic acid, suggesting that oxido-reduction may have occured in urocanic acid molecule as in the anaerobic enzymic process.

The role of L-ascorbic acid may be the formation by aeration of monodehydroascorbic acid which is the intermediate to dehydroascorbic acid and has the radical semiquinone type. (*10*, *11*, *12*)

SUMMARY

1. The degradation of imidazole derivatives by aeration in the presence of L-ascorbic acid was studied. It was found that the amino acids formed have the structures corresponding to the starting imidazole derivatives.

2. While hydroxymethylimidazole was hardly attacked by H_2O_2, histidine is easily oxidized by H_2O_2 as well as by aeration in the presence of ascorbic acid. Aspartic acid was detected and isolated from the oxidation products. The effect of ascorbic acid cannot be attributed simply to the action of H_2O_2 secondarily formed in the autoöxidation.

3. The effects of imidazole derivatives on the autoöxidation of L-ascorbic acid (preceding paper) were discussed taking the reactivity as the chain breaker into account.

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ON THE INFLUENCE OF THE DEFICIENCY OF ASCORBIC ACID UPON THE ACTIVITY OF THE COMPLEMENT IN THE SERUM

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On the relation between the concentration of ascorbic acid and the activity of the complement in the serum, a number of experiments have been performed but their results are not consistent. Simola and Brunius (*1*) and Marsh (2fifi) reported that by the deficiency of the ascorbic acid, the complement activity decreased remarkably and Ecker and his coworkers (*3*) stated that so far as the concentration of ascorbic acid in the serum is below than one mg. per cent, complement activity also decreased keeping pace with the concentration of the ascorbic acid and that the complement activity of the serum of guinea-pigs suffering from scurvey can be restored by injection of ascorbic acid. Anderson (*4*) stated ascorbic acid to be one component of the complement in the serum.

On the other hand Spink, Agnew and Mickelson (*5*) reported that the decrease of the concentration of the ascorbic acid in serum did not accompany the decrease of the complement activity of the serum, and also *in vitro* the addition of ascorbic acid to serum did not increase its complement activity.

According to the present studies, the complement consists of four components, namely midpiece, endpiece, the 3rd and the 4th component. By the studies of Heiderberger and Mayer (*6*) it has become clear that the value of the complement may be determined by the concentration of the minimum constituent of the complement, the 3rd component, therefore merely by the determination of them coplement value we can not decide th change of each component of the complement.

From this standpoint we experimented to know the relation between the concentration of ascorbic acid and each component of the complement in serum, and if any effect can be seen on the complement

value, whether ascoric acid itself or pathological constituents in the serum accumulated as the results of the deficiency of ascorbic acid exert this effect.

EXPERIMENT

(1) *Experimental Animals*—Guinea-pigs were used as experimental animals. A group of healthy male guinea-pigs accustomed to the test diet deficient in ascorbic acid were injected with 50 mg. of ascorbic acid every day, for about one week. Then the animals devided into two groups and one group was continued to be fed and injected on the same way and the another group was kept on the same diet was without injection of ascorbic acid. The symptomes of the deficiency of ascorbic acid appeared generally after three or four weeks. Then the amount of each of the complement was determined on the serums of two groups of animals.

The constituent of the test diet deficient for ascorbic acid was as follows :

Wheat bran (Fusuma in Japanese)	100 g.
Wheat powder	50 g.
Fish meat powder	50 g.
Soya bean oil	6 g.
Cod liver oil	2 g.
Sodium chloride	2 g.

Animals were allowed to eat as they wish.

(2) *Determination of Ascorbic Acid*—Blood taken from heart by puncture was stand 37° for one hour and the serum separated was used for the determination of ascorbic acid by the method of 2,4-dinitrophyenyl hydrazin according to Fujita and Teruuchi (*7*).

(3) *Determination of the Complement Value*—The value of the complement was determined by the degree of hemolysis of sheep red corpuscles caused by the test serum in the presence of sufficient amount of hemolysins. The degree of the hemolysis was estimated according to Heidelberger and Mayer's method (*6*).

In the series of test tubes 0.1, 0.2, 0.3, 0.4, 0.5 ml. of th test serum diluted to 100 times were taken, and 0.25 ml. of suspension of sensitized sheep corpuscles, which contained difinite amount of hemolysin, were added to each tube. Then physiological saline solution was added to each tube as its volume bacame 1.5 ml.

After an incubation at 37° for one hour the supernatant liquid of each tube was separated by centrifugation and the concentration of hemoglobin in each of them was determined with photoelectric colorimeter. The concentration of hemoglobin in a tube in which the complete hemolysis occurred was taken as 100 per cent.

For the determination of each component of the complement, the serum, in which only the component was inactivated and others were contained sufficiently, was prepared, to which sensitized corpuscles and these serum were added and the degree of hemolysis was observed after an incubation.

The relation between complement value and degree of hemolysis is indicated in Fig. 1.

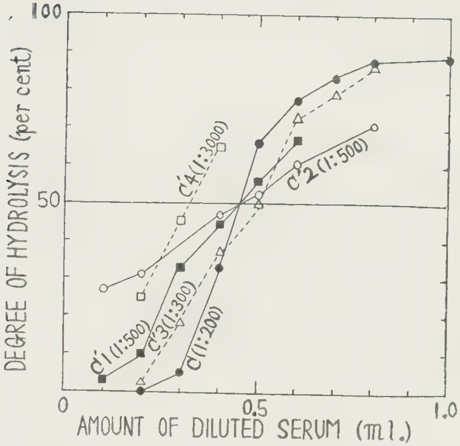

FIG. 1. Relation between the Value of Complemet and degree of hemolysis.

From this results we can see the parallelism between the complement value and the degree of hemolysis in the range from 40 to 60 per cent. The amount of complement which is necessary to cause the 50 per cent hemolysis is determined and this amount of complement was teaken as one unit, and the number of units contained in 1.0 ml. of the original serum were calculated.

The method of inactivation of each component of the complement is as follows.

(*i*) *Inactivation of the Midpiece* (C' 1)—The serum was diluted with distilled water to ten times and carbon dioxide gas was bubbled through it. By centrifugation a supernatant liquid, the albumin fraction, was separated. This fraction contained the endpiece and the 4th component. To 0.2 ml. of this fraction the same amount of 10 per cent serum heated at 56° for 30 minutes, which contained the 3rd and 4th component, was added. This mixture lacked the midpiece.

(*ii*) *Inactivation of the Endpiece* (C' 2)—A sediment of globulin fraction, which was separated from the serum by bubbling of carbon dioxide gas through it, was dissolved in physiological saline solution of ten times of the original serum. This solution contained the midpiece and 3rd component. To 2.0 ml. of this solution the same amount of 1.0 per cent serum heated at 56° for 30 minutes was added. This mixture lacked the endpiece.

(*iii*) *Inactivation of the 3rd Component* (C' 3)—Brewers yeast was washed with acetone and ether successively and then extracted with water throughly and dried. The serum was mixed with the same amount of 10 per cent of the suspension of the yeast thus treated and incubated for 90 minutes at 37° with occasional shakings. The mixture was diluted to 5 times with saline solution. This lacked the 3rd component.

(*iv*) *Inactivation of the 4th Complnent* (C' 4)—A 0.25 ml. of $N/6.5$ ammonium hydro-

xide solution was added to 10 ml. of serum and the mixture was allowed to stand at 37° for 75 minutes. It was then neutralysed with $N/6.5$ hydrochloric acid and diluted with 8.5 ml. of physiologial saline solution. This lacked the 4th component.

RESULTS

I. Contents of Ascorbic Acid and the Components of the Complement of the Normal Guinea-pigs Serum—A group of guinea-pigs was fed on the test diet as shown above and injected with 50 ml. of ascorbic acid every day for one week or more and at the end of this period the contents of ascorbic acid and each component of complement in the serum were estimated. The resuls are as follows. (See Table I)

TABLE I

Concentration of Ascorbic Acid and Each Component in Normal Guinea-pigs Serum

No. of animal		Body weight g.	Asc. acid *mg./dl.*	C *units/ml.*	C'1 *units/ml.*	C'2 *units/ml.*	C'3 *units/ml.*	C'4 *units/ml.*
	Before experiment	520	1.90	610	1850	1250	690	8000
1	Test diet+V.C	500	0.62	590	1850	1250	590	7000
	"	540	0.87	620	2100	800	710	11000
	Before experiment	510	0.84	760	2000	1250	830	9000
2	Test diet+V.C	550	0.43	720	2100	1400	620	7000
	"	540	0.73	710	2500	1500	720	10000
	Before experiment	540	1.06	400	1500	800	410	11000
3	Test diet+V.C	550	0.74	630	1400	1250	690	13000
	"	580	0.48	580	2300	1900	640	8000
	Before experiment	650	0.68	560	1500	1100	690	8000
4	Test diet+V.C	670	0.62	670	1800	1500	710	10000
	"	740	0.43	530	2500	1800	710	11000
	Before experiment	560						
5	Test diet+V.C	500	0.31	540	2000	2100	710	10000
	"	480	0.38	550	2500	2200	710	9000
	Before experiment	560	1.12 ±0.30	580 ±130	1700 ±220	1100 ±180	650 ±150	9000 ±2300
Averages	Test diet+V.C	550	0.54 ±0.12	610 ± 40	1800 ±240	1500 ±320	660 ± 50	9000 ±2300
	Test diet+V.C	580	0.58 ±0.19	600 ± 80	2400 ±160	1600 ±480	710 ± 40	1000 ±1200

II. Contents of Ascorbic Acid and Each Component of the Complement of Guinea-pigs Suffering from Lack of Ascorbic Acid.—A group of guinea-pigs was fed with the test diet wih or without the injection of ascorbic acid for a period of 7 to 21 days. Before the feeding and at the end of this period the serum were analysed. The results are indicated in Table II.

As indicated in the above table, by the feeding guinea-pigs on the

TABLE II

Concentration of Ascorbic Acid and Each Component of Complement in the Serum of Guinea-pigs Suffering from Lack of Ascorbic Acid

No. of animal		Body wt. g.	Asc. acid *mg./dl.*	C *units/ml.*	C'1 *units/ml.*	C'2 *units/ml.*	C'3 *units/ml.*	C'4 *units/ml.*
11	Before experiment	600	1.37	530	2000	1100	800	8000
	Test diet+V.C	580	0.62	420	2000	1350	610	7000
	Test diet	530	0.17	510	2100	1150	550	9000
	Test diet+V.C	560	0.38	450	1600	1150	640	8000
12	Before experiment	480	1.90	750	1700	1800	700	10000
	Test diet+V.C	470	0.80	650	2300	1600	710	11000
	Test diet+V.C	480	0.20	660	2100	2000	670	10000
13	Before experiment	560	0.46	530	1500	1200	540	7000
	Test diet+V.C	540	0.38	540	1700	1300	570	7000
	Test diet	520	0.26	510	2100	1200	540	8000
	Test diet+V.C	560	0.86	560	2000	1300	640	9000
14	Before experiment	560	0.53	530	1700	800	800	6000
	Test diet-V.C	540	0.46	540	1900	1400	870	7000
	Test diet	520	0.20	650	2400	1360	1000	8000
	Test diet+V.C	580	0.62	580	1900	1700	900	10000
15	Before experiment	620	0.68	670	2000	1500	830	8000
	Test diet+V.C	640	0.46	670	1900	1600	830	9000
	Test diet	640	0.06	570	1500	1450	660	7000
	Test diet+V.C	640	0.55	570	2000	1800	800	8000
16	Before experiment	620	1.29	770	2000	1500	870	10000
	Test diet+V.C	640	0.53	690	2000	1600	830	10000
	Test diet	640	0.02	730	2000	1450	780	8500
	Test diet+V.C	680	0.72	690	1800	1800	800	8000
Mean values	Before experiment	570	0.87 ±0.55	630 ±150	1800 ± 200	1300 ± 320	750 ±100	8000 ±1500
	Test diet+V.C	570	0.54 ±0.13	590 ±130	2000 ± 180	1500 ± 170	740 ±120	8500 ±1600
	Test diet	550	0.15 ±0.08	610 ± 80	2100 ± 300	1400 ± 310	700 ±160	8400 ± 900
	Test die-+V.C	580	0.63 ±0.16	570 ± 80	1900 ± 320	1600 ± 320	750 ±110	8600 ± 700

test diet with injection of ascorbic acid every day, the concentration of ascorbic acid in the serum decreased to some degree, but an amount of each component of the complement remained unchanged.

From these results it was clarified that though the ascorbic acid contents of the serum decrease by feeding with diet deficient of ascorbic acid, each component of the complement of serum underwent no remarkable change.

In this experiment the animals developed none of such symptoms as lankness, swelling of joint, bleeding in mucous membrane, and excretion of bloody excrements.

In the next experiment the animals were fed with diet deficient in ascorbic acid for longer period until the above-described symptoms developed, and the same investigations were performed with their serum. The results were as follows. (Table III)

As can be seen from this table, no change in the contents of each

TABLE III

Contents of Ascorbic Acid and Its Component of the Serum of Guinea-pigs Suffering from Scurvy

No. of animal		Body wt. g.	Asc. acid. *mg./dl.*	C *units/ml.*	C'1 *units/ml.*	C'2 *units/ml.*	C'3 *units/ml.*	C'4 *units/ml.*
	Before experiment	720	1.70	420	2300	1250	500	8000
21	Test diet+V.C	700	0.41	400	1800	1900	570	10500
	Test diet	480	0.31	450	2000	1600	600	10000
	Before experiment	660	1.72	680	2900	2500	670	10000
22	Test diet+V.C	600	0.55	720	2000	2500	720	9000
	Test diet	440	0.24	710	2500	2500	710	9000
	Before experiment	460	1.30	560	1900	2200	730	11000
23	Test diet+V.C	440	0.55	580	1900	2200	830	12000
	Test diet	330	0.31	550	2000	2000	800	10000
	Before experiment	580						
24	Test diet+V.C	560	0.52	470	1250	1200	500	11000
	Test diet	380	0.38	350	1000	700	500	10000
	Before experiment	610	1.00	530	1750	2300	740	9500
25	Test diet+V.C	540	0.31	550	1700	2000	710	9000
	Test diet	420	0.14	700	2000	1600	900	8000
	Before experiment	590	1.42	550	2200	2050	660	9600
			±0.31	± 90	±450	±480	±100	±1100
Mean values	Test diet+V.C	570	0.47	540	1750	1950	690	10300
			±0.09	±110	±300	±430	± 90	±1200
	Test diet	410	0.28	550	1900	1900	700	9400
			±0.08	±140	±440	±370	±140	±1000

component of complement could be observed, although the animals developed severe scurvic symptoms and showed a remarkable decrease in ascorbic acid content of serum.

III. Effect of Addition or Reduction of Ascorbic Acid in the Serum Upon Complement Value in Vitro—Ascorbic acid was dissolved in guinea-pig's serum in a concentration of about 5 mg. per dl. *in vitro*, and the effect on the complement activity was examined. The results were as follows.

TABLE IV

Effect of Ascorbic Acid upon the Complement Value in Vitro

No. of animal		Concentration of asc. acid *mg./dl.*	C *units/ml.*	C′1 *units/ml.*	C′2 *units/ml.*	C′3 *units/ml.*	C′4 *units/ml.*
11	After addition of asc. acid	0.17	510 480	2100 2100	1150 1200	550 580	9000 9000
15	After addition of asc. acid	0.06	570 600	1500 1600	1200 1160	660 640	7000 7000
11	After addition of asc. acid	0.02	770 780	2000 2000	1450 1450	780 790	8500 8500

As indicated in this experiment no effect could be observed upon complement value also *in vitro* by the addition of ascorbic acid to the normal serum. Next, ascorbic acid in the normal serum was reduced by the addition of a $CuCl_2$ solution *in vitro*, and the effect of the low content of the acid upon complement value was investigated. To 1 ml. of normal guinea-pig serum 0.1 ml. of $M/100$ $CuCl_2$ solution was added

TABLE V

Effect of Reduction of Ascorbic Acid in Serum in Vitro upon Complement Value

	Concentration of ascorbic acid *mg./dl.*	C *units/ml.*	C′1 *units/ml.*	C′2 *units/ml.*	C′3 *units/ml.*	C′4 *units/ml.*
After reduction of asc. acid	0.80 0.23	350 350	2000 1800	1450 1450	740 760	9000 9500
After reduction of asc. acid	0.62 0.21	460 460	2600 2400	1600 1500	850 800	12000 12000

and incubated at 37° for 1 hour. The reduction of ascorbic acid in the serum was apparent.

It was clarified that no effect could be seen upon the content of the complement by the destruction of ascorbic acid in the normal serum.

SUMMARY

1. Each component of the complement of guinea-pig's serum in which ascorbic acid was reduced remarkably by feeding with the diet deficient in ascorbic acid, or even of the serum of animals which were suffering from severe scurvy, remained without any reduction.

2. Addition of ascorbic acid to the serum of scorbutic guinea-pigs *in vitro* gave no change in the content of each component of the complement.

3. Lowering of ascorbic acid contents in the normal guinea-pig's serum by the addition of $CuCl_2$ *in vitro*, resulted in no change in each of its component of the complement.

From these results it may be said that any component of complement has no relatin with ascorbic acid directly.

The authors wish to express their sincerest thanks to Prof. Shiro Fujimura for his kind directions in carrying out this work.

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ON THE INFLUENCE OF CERTAIN SALTS UPON THE ACTIVITY OF THE COMPLEMENT

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The fact that various inorganic ions inhibit the activity of complement was reported by many investigators. Hektön and Rüdinger (1) demonstrated that Mg, Ca, Ba, Sr and SO_4 ions possessed the anti-complementary activity, and Manwaring (*2*) stated that the influence of these ions upon the complement could be removed by precipitating them with suitable salts. Michaelis and Skwirsky (*3*) showed that in a medium containing NaH_2PO_4 and Na_2HPO_4 the hemolytic reaction did not occur and that, in this mixture which suppressed the action of endpiece, midpiece could combine with sensitized corpuscles and the activity of endpiece could be restored by neutralizing the excess of the acid phosphate with Na_2HPO_4.

Lately, besides the mid- and endpiece, the other two components, such as the third and fourth components, were found by Omorokow (*4*), Ritz (*5*) and Grdon (*6*) respectively. It appeared, therefore, of interest to investigate the influence of salts upon the complement concerning with each of these four components.

In the former studies, the salt solutions were used in various concentrations in general, but it must be mentioned that when the sensitized corpuscles are suspended in hypo- or hypertonic solution they are in the different condition than the normal. In the present study, therefore, the solutions of various salts, isotonic with the physiological saline solution, were used.

EXPERIMENTAL AND RESULTS

Method of the Test of Complement Action

As the complement guinea-pig serum was used an the amount sufficient to hemolysis 0.25 ml. of 3 per cent suspension of sheep red corpuscles in a presence of 5 units of hemolysins, is taken as one unit. The test of the activity of complement was based on the hemolytic reaction.

In the series of test tubes, 0.25 ml. of 3 per cent suspension of sheep sensitized corpuscles was taken and the complement sera diluted to ten times or the solutions of the component of complement in an amount of 0.05, 0.1, 0.15,......0.5 ml. were added and each mixture was incubated at 37° for one hour, with occasional shakings. At the end of this period the degree of hemolysis was examined.

A. Anticomplementary Action of Cations

I. Determination of the Anticomplementary Action of Cations—As cations K, Ca, Ba, Sr and Mg were used. Solutions of 0.165 *M* KCl, 0.117 *M* $CaCl_2$, 0.110 *M* $BaCl_2$, 0.110 *M* $SrCl_2$ and 0.114 *M* $MgCl_2$ which were isotonic with physiological saline solution, were prepared. The complement serum was diluted to ten times with physiological saline solution, and a definite portion of the latter was replaced with the salt solutions above mentioned. Thus complement solutions which were diluted to ten times, containing each cation in a definite concentration, and isotonic with physiological saline solution, were obtained.

With these complement solutions and sensitized corpuscles, hemolyzing tests were performed. The results are shown in Table I. In the table, cases where no hemolysis occurs are indicated as (−), and complete hemolysis as (卌), while for intermediate cases the signs (±), (+), and (++) are used.

In cases of KCl and $MgCl_2$, sensitized corpuscles were suspended in each isotonic solution.

As can be seen from these results, Ca^{++}, Ba^{++}, Sr^{++} and Mg^{++} had a strong anticomplementary action while K^{+} indicated no effect on the complementary action within the limits of a concentration of 0.165 *M*.

These effects of the ions on the complement can be illustrated in Fig. 1. indicating the relation between the amount of complement, which is inactivated completely, and concentrations of the salts. As indicated in Table I, 0.1 ml. of guinea-pig serum was used in this experiment, which contained one unit of complement.

These results indicate that the sufficient concentrations of the salts which inhibited the action of 5 units of complement were 0.034 *M* in $BaCl_2$, 0.031 *M* in $SrCl_2$ and 0.076 *M* in $MgCl_2$, which, sufficient for 3 units of complement, was 0.054 *M* in $CaCl_2$.

In the following experiments, therefore, the isotonic solutions, containing each of these salts in the concentrations shown above, were used.

II. Effect of the Removal of the Salts—The cations, such as Ca, Ba, Sr and Mg, had an anticomplementary action remarkably as shown in the previous experiment. To determine whether the effect of the cations was due to the essential change of the complement or only to the passing phenomena by the presence of the ions, the following experiments were performed.

The serum, diluted to ten times with each salt solution in the above concentration, was incubated at 37° for one hour and then dialyzed against running water to remove salts completely. After dialyzed, volumes of each complement solution were adjusted to the original volumes and sodium chloride was dissolved to a concentration of 0.85 per cent and their complementary activities were examined. The results are as follows.

TABLE I

Anticomplementary Action of the Cations

Amount of complement (1:10) *ml.*		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (each salt solution) 0.5
Control test (diluted with NaCl)		++	+++	+++	+++	+++	+++	+++	+++	−
KCl	KCl : NaCl									
	3 : 6	++	+++	+++	+++	+++	+++	+++	+++	−
	6 : 3	++	+++	+++	+++	+++	+++	+++	+++	−
	9 : 0	++	+++	+++	+++	+++	+++	+++	+++	−
$CaCl_2$	$CaCl_2$: NaCl									
	3 : 6	−	−	±	+	++	+++	+++	+++	−
	6 : 3	−	−	−	−	±	+	+++	+++	−
	7.5 : 1.5	−	−	−	−	−	−	++	+++	−
$BaCl_2$	$BaCl_2$: NaCl									
	2 : 7	−	−	−	+	++	+++	+++	+++	−
	3 : 6	−	−	−	−	−	−	+	+++	−
	4.2 : 4.8	−	−	−	−	−	−	−	−	−
$SrCl_2$	$SrCl_2$: NaCl									
	2 : 7	−	−	−	±	++	+++	+++	+++	−
	3 : 6	−	−	−	−	−	−	+	+++	−
	3.8 : 5.2	−	−	−	−	−	−	−	−	−
$MgCl_2$	$MgCl_2$: NaCl									
	3 : 6	−	−	±	+++	+++	+++	+++	+++	−
	6 : 3	−	−	−	−	−	++	+++	+++	−
	9 : 0	−	−	−	−	−	−	−	±	−

For the removal of Ca^{++} and Ba^{++} from the complement solution, the precipitation by the addition of sodium oxalate or sodium sulphate was also attempted. To the complement solution containing Ca^{++} or Ba^{++}, equivalent amount of sodium oxalate or sodium sulphate was added, respectively, and the clear supernatant solutions were obtained by centrifugation. Using these solutions, hemolyzing tests were performed and the same results as above illustrated were obtained.

As can be seen in Tables II and III, the activity of the complement was restored completely by the elimination of the salts.

It was, therefore, shown that the anticomplementary action of the salts was not based on the destruction of the complement, but on the passing phenomena owing to the presence of the salts an that by the elimination of the salts by dialysis or precipitating with the suitable salts, the activity of complement could be restored.

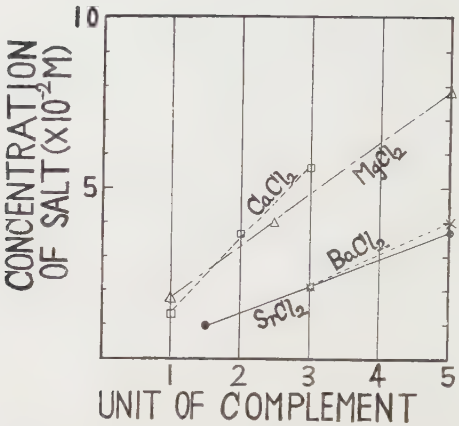

FIG. 1. Relation between the anticomplementary concentrations of salts and the unit of complement.

TABLE II

Effect of the Removal of the Salts by Dialysis

	Amount of complement (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	Before dialysis	+	+++	+++	+++	+++	+++	+++	+++	—
	After dialysis	—	++	+++	+++	+++	+++	+++	+++	—
$CaCl_2$	Before dialysis	—	—	—	—	—	—	—	—	—
	After dialysis	+++	+++	+++	+++	+++	+++	+++	+++	—
$BaCl_2$	Before dialysis	—	—	—	—	—	—	—	—	—
	After dialysis	—	+	+++	+++	+++	+++	+++	+++	—
$SrCl_2$	Before dialysis	—	—	—	—	—	—	—	—	—
	After dialysis	—	++	+++	+++	+++	+++	+++	+++	—
$MgCl_2$	Before dialysis	—	—	—	—	—	—	—	—	—
	After dialysis	—	+	+++	+++	+++	+++	+++	+++	—

III. Effect of the Salts on the Combination of Corpuscles and Hemolysins—As it became clear that the effect of salts on the complementary action did not base on the destruction of the complement, the present experiment was performed to decide whether the salts

TABLE III

Effect of the Removal of the Salts by Suitable Salts

Amount of Complement (1 : 10) *ml.*			0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)		1 :10	++	+++	+++	+++	+++	+++	+++	+++	—
		1 :20	—	++	+++	+++	+++	+++	+++	+++	—
$CaCl_2$	Treated with oxalate		—	—	—	—	—	—	—	—	
			—	±	+++	+++	+++	+++	+++	—	
$BaCl_2$	Treated with sulphate		—	—	—	—	—	—	—	—	
			—	—	±	++	+++	+++	+++	+++	

TABLE IV

Effect of the Salts on the Combination of Corpuscles and Hemolysins

Amount of complement (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.05	Control (NaCl) 0.5
Control test (NaCl)	+	+++	+++	+++	+++	+++	+++	+++	—
$CaCl_2$	—	+++	+++	+++	+++	+++	+++	+++	
$BaCl_2$	±	+++	+++	+++	+++	+++	+++	+++	
$SrCl_2$	—	+++	+++	+++	+++	+++	+++	+++	
$MgCl_2$	±	+++	+++	+++	+++	+++	+++	+++	

interfered the combination of the corpuscles and hemolysins or not, which was the first step of the phenomena in immune hemolysis.

To each solution of the salts, containing enough concentrations to inhibit the complementary action, red corpuscles and hemolysins were added and incubated at 37° for 1 hour. After the incubation, the corpuscles were separated by centrifugation and washed with physiological saline solution and suspended in physiological saline solution in a concentration of 3 per cent. With the suspension thus obtained and complement serum, hemolyzing test was performed and following results were obtained. (Table IV). At the same time a control test was performed in which the corpuscles were sensitized with hemolysins in absence of the salts.

The result indicates that the salts did not interfere the combination of the corpuscles

TABLE V

Effect of the Salts on the Combination of Midpiece and Sensitized Corpuscles

3rd component albumin fraction (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	+	卌	卌	卌	卌	卌	卌	卌	—
$CaCl_2$	—	—	—	—	—	—	—	—	—
$BaCl_2$	—	—	—	—	—	—	—	—	—
$SrCl_2$	—	—	—	—	—	—	—	—	—
$MgCl_2$	—	—	—	—	—	—	—	—	—

TABLE VI

Effect on the Combination of the 4th Component and Sensitized Corpuscles

Serum treated with NH_4OH (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	—	+	卌	卌	卌	卌	卌	卌	—
$CaCl_2$	—	—	—	—	—	—	—	—	—
$BaCl_2$	—	—	—	—	—	—	—	—	—
$SrCl_2$	—	—	—	—	—	—	—	—	—
$MgCl_2$	—	—	—	—	—	—	—	—	—

and hemolysins.

IV. Effect of the Salts on the Combination of Complement and Sensitized Corpuscles—From the preceeding experiments it can be said that the mechanism of the anticomplementary action of the salts is based neither on the destruction of the complement nor on the interference of the combination of the corpuscles and hemolysins. Then, the author attempted to know the effect of the salts on the combination of complement and sensitized corpuscles.

In the recent studies, it has been found that the complement consists of four components, such as midpiece, endpiece, the 3rd and the 4th component. According to Ueno's studies (*7*) it is clear that the sensitized corpuscles combine at first with the midpiece, and 4th component and thereafter endpiece, finally the 3rd component. At

TABLE VII

Effect on the Combination of the Endpiece and Sensitized Corpuscles

3rd component (1 : 10) ml.	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	–	+	+	++	+++	+++	+++	+++	–
$CaCl_2$	–	–	–	–	–	–	–	–	–
$BaCl_2$	–	–	–	–	–	–	–	–	–
$SrCl_2$	–	–	–	–	–	–	–	–	–
$MgCl_2$	–	–	–	–	–	–	–	–	–

TABLE VIII

Effect on the Combination of the third Component and Sensitized Corpuscles

3rd component (1 : 10) ml.	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	–	–	+	++	+++	+++	+++	+++	–
$CaCl_2$	–	–	–	–	–	–	–	–	–
$BaBl_2$	–	–	–	–	–	–	–	–	–
$SrCl_2$	–	–	–	–	–	–	–	–	–
$MgCl_2$	–	–	–	–	–	–	–	–	–

which part of this mechaism do the salts exert their inhibiting action?

The following experiments were performed to solve this problem.

The methods of preparation of the component of complement were as follows.

(1) *Preparation of the Midpiece and Endpiece*—To 1 ml. of serum 0.25 ml. of $N/6.5$ NH_4OH was added, and the mixture was incubated at 37° for 75 minutes and then neutralized with 0.25 ml. of $N/6.5$ HCl. After neutralization, 1 ml. of a 10 per cent suspension of Brewer's yeast—extracted with acetone and ether successively and then washed with water throughly and dried—in saline was added to it, and the mixture was incubated at 37° for 90 minutes with occasional shakings. At the end of this period the supernatant liquid was separated by centrifugation.

By this treatment the 4th and 3rd components were destroyed. After the addition of 7.5 ml. of distilled water to it, carbon dioxide gas was bubbled through this serum, and then by centrifugation the precipitate and supernatant were obtained. The super-

TABLE IX

Anticomplementary Action of the Anions

Amount of complement (1 : 10) *ml.*		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (each solution) 0.5
Control test (diluted with NaCl)		++	+++	+++	+++	+++	+++	+++	+++	—
Na_2SO_4	Na_2SO_4 : NaCl									
	3 : 6	—	—	—	+	++	+++	+++	+++	—
	6 : 3	—	—	—	—	+	+++	+++	+++	—
	9 : 0	—	—	—	—	—	—	+	+++	—
$NaNO_3$	$NaNO_3$: NaCl									
	9 : 0	++	+++	+++	+++	+++	+++	+++	+++	—
$NaCH_3COO$	$NaCH_3COO$: NaCl									
	9 : 0	++	+++	+++	+++	+++	+++	+++	+++	—
KH_2PO_4	KH_2PO_4 : NaCl									
	4 : 5	—	—	++	+++	+++	+++	+++	+++	—
	4.5 : 4.5	—	—	—	—	—	++	+++	+++	—
	5 : 4	—	—	—	—	—	—	—	—	—
	5 : 4 (pH 7.0)	+++	+++	+++	+++	+++	+++	+++	+++	—
Na_2HPO_4	Na_2HPO_4 : NaCl									
	5 : 4 (pH 9.0)	+++	+++	+++	+++	+++	+++	+++	+++	—
	5 : 4 (pH 5.0)	—	—	—	—	—	—	—	—	—
Control (NaCl) pH 5.0		+	+++	+++	+++	+++	+++	+++	+++	—

natant liquid, to which sodium chloride was added to a concentration of 0.85 per cent, contained only the endpiece. The precipitate was dissolved in 10 ml. of physiological saline solution, and this solution contained only the midpiece. Both solutions diluted 10 times were thus obtained.

(2) *The Preparation of the 3rd Component*—The serum, treated with ammonia solution in the same way described as above, was heated at 56° for 30 minutes to inactivate the mid- and endpieces. This was diluted to 10 times and used.

(3) *The Preparation of the 4th Component*—The albumin fraction of the serum, which was obtained by means of bubbling through of carbon dioxide gas (*8*), was heated at 56° for 30 minutes to destroy the endpiece.

(*i*) *Effect of the Salts on the Combination of Midpiece an Sensitized Corpuscles*—An

Table X

The Effect of the Removal of the Salts

Amount of complement (1 : 10) *ml.*		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control (NaCl)	Before dialysis	++	+++	+++	+++	+++	+++	+++	+++	—
	After dialysis	—	++	+++	+++	+++	+++	+++	+++	
Na_2SO_4	Before dialysis	—	—	—	—	—	—	+	++	
	After dialysis	—	++	+++	+++	+++	+++	+++	+++	
KH_2PO_4	Before dialysis	—	—	—	—	—	—	—	—	
	After dialysis	—	+++	+++	+++	+++	+++	+++	+++	

Table XI

Effect on the Combination of Corpuscles and Hemolysins

Amount of complement (1 :10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Contr ol (NaCl) 0.5
Control (NaCl)	+	+++	+++	+++	+++	+++	+++	+++	—
No_2SO_4	++	+++	+++	+++	+++	+++	+++	+++	—
KH_2PO_4	+	+++	+++	+++	+++	+++	+++	+++	—

Table XII

Effect on the Combination of the Midpiece and Sentisitized Corpuscles

3rd component albumin fraction *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	—	++	+++	+++	+++	+++	+++	+++	—
Na_2SO_4	—	—	—	—	—	—	—	—	—
KH_2PO_4	—	—	—	—	—	—	—	—	—

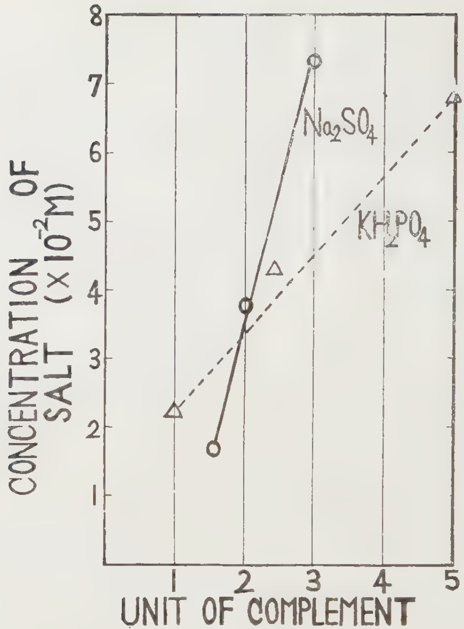

FIG. 2. Relation between anticomplementary concentration and unit of complement.

TABLE XIII

Effect on the Combination of 4th Component and Sensitized Corpuscles

Serum, treated with NH_4OH (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control (NaCl)	—	++	+++	+++	+++	+++	+++	+++	—
Na_2SO_4	—	—	—	—	—	—	—	—	—
KH_2PO_4	—	+++	+++	+++	+++	+++	+++	+++	—

aliquot of 10 ml. of sensitized corpuscles suspension was centrifuged and to the separated corpuscles, 10 ml. of the midpiece solution, which was diluted with each of the salt solution, was added. After incubation at 37° for 30 minutes, the corpuscles were collected by centrifugation and suspended again in 10 ml. of the physiological saline solution. To

TABLE XIV

Effect on the Combination of the Endpiece and Sensitized Corpuscles

3rd component (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	−	+	+	++	+++	+++	+++	+++	−
Na_2SO_4	−	−	−	−	−	−	−	−	−
KH_2PO_4	−	−	−	−	−	−	−	−	−

TABLE XV

Effect on the Combination of the 3rd Component and Sensitized Corpuscles

3rd component (1 : 10) *ml.*	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	Control (NaCl) 0.5
Control test (NaCl)	−	−	+	++	+++	+++	+++	+++	−
Na_2SO_4	−	−	−	−	−	−	−	−	−
KH_2PO_4	−	−	−	−	−	−	−	−	−

this endpiece suspension, the 3rd and the 4th components wer added separately and degrees of hemolysis were examined after the incubation. If the sensitized corpuscles can combine with the midpiece in the presence of the salts, hemolysis must occur by the addition of the other components.

The results were as follows.

As shown in Table V, the combination of the midpiece and sensitized corpuscles were interfered by th presence of the salts remarkably.

(ii) Effect of the Salts on the Combination of the 4th Componet and Sensitized Corpuscls— A 10 ml. portion of the suspension of the sensitized corpuscles was centrifuged. This separated corpuscles were mixed with 10 ml. of the midpiece solution and incubated at 37° for 30 minutes. After incubation, corpuscles were separated and 10 ml. of the 4th component solution was added to it. The mixture was diluted with each salt solution and incubated at 37° for one hour. After incubation, the separated corpuscles were resuspended again in the physiological saline solution and then the mixture of endpiece and the 3rd component was added to it. Degree of hemolysis was examined after incubation for 1 hour at 37°.

The results, sown in Table VI, indicate that each salt interfered the combination of the 4th component and sensitized corpuscles which had been combined with midpiece.

(*iii*) *Effect on the Combination of the Endpiece and Sensitized Corpuscles*—Sensitized corpuscles, which had been combined with the midpiece and the 4th component in the same way as described above, were suspended in the endpiece solution, containing each salt solution, and the suspensions were allowed to stand at 37° for 1 hour. Then the corpuscles were separated from the liquid and suspended in the physiological saline solution, and the 3rd component solution was added to it. Degree of hemolysis was examined after incubation. As shown in Table VII, each salt also interfered the combination of endpiece and sensitized corpuscles.

(*iv*) *Effect on the Combination of the 3rd Component and Sensitized Corpuscles*—Sensitized corpuscles, separated by centrifugation, were suspended in the diluted serum which had been inactivated by Brewer's yeast, and each suspension was incubated at 37° for 1 hour. By this way, sensitized corpuscles were combined with the midpiece, the 4th component and the endpiece. Then, the corpuscles were separated from the solution and suspended again in physiological saline solution. To this corpuscles suspension, the 3rd component solution, diluted with each salt solution, was added and the degree of hemolysis was examined. The following results were obtained.

Each salt interfered the combination of the 3rd component and the sensitized corpuscles which had been already united with other three components.

Thus, it was clearly shown that the inorganic salts, such as $CaCl_2$, $SrCl_2$ and $MgCl_2$, interfered the combination of sensitized corpuscles and each of the component of the complement.

B. Anticomplementary Action of the Anions

I. Determination of the Anticomplementary Action of the Anions—As anion, $SO^{--}{}_4$, NO_3^-, $PO^{--}{}_4$ and CH_3COO^- were examined. Solutions of 0.134 *M* Na_2SO_4, 0.153 *M* Na NO_3, 0.174 *M* KH_2PO_4, 0.129 *M* Na_2HPO_4 and 0.214 *M* $NaCH_3COO$ were prepared. The solutions were isotonic with the physiological saline solution. The effect of the salts was investigated below these concentrations.

The anticomplementary action of these anions were studied in the same way as in the previous experiment. The results were as follows.

In cases of Na_2SO_4, $NaNO_3$ and CH_3COONa, the sensitized corpuscles were suspended in each solution.

As shown in Table IX, SO_4^{--} and $H_2PO^-{}_4$ had the remarkable anticomplementary action, but the action of KH_2PO_4 disappeared when the solution was neutralized with sodium hydroxide, while the action of Na_2HPO_4 appeared when its solution was acidified to pH 5.0 with diluted hydrochloric acid. These phenomena were considered to be due to the change of dissociation of phosphate, but not to the effect of the change of pH value.

As indicated in Table IX, 0.1 ml. of complement solution, used in this experiment, corresponded to one unit.

The relation between anticomplementary concentrations of salts and the amounts of complement is shown in Fig. 2.

Fig. 2 shows that the concentration of KH_2PO_4 solution, which was sufficient to

inhibit the action of 5 units of complement, was 0.0645 *M*, and that the inhibitory power of Na_2SO_4 was not so strong that concentration to inhibit the activity of 3 units of complement was 0.0732 *M*.

To interfere the action of complement in the following experiments, the solutions of these concentrations were used.

II. Effect of the Removal of the Salts—To determine whether the anticomplementary action of the anions was due to the essential change of the complement or only the passing phenomena by the presence of the salts, following experiment was performed in the same way as described in the case of the cations.

The result showed that the anticomplementary action of the anions was but the passing phenomena by the presence of them, as in the case of cations.

III. Effect of the Anions on the Combination of Corpuscles and Hemolysins—The effect of the anions on the combination of corpuscles and hemolysins were investigated in the same method as the case of the cations.

The result, shown in Table XI, indicated that the anions had no effect on the combination of corpuscles and hemolysins.

IV. Effect on the Combination of Sensitized Corpuscles and the Complement—Complement was fractionated in 4 components by the methods described in the previous section. The effects of the anions on the combination of sensitized corpuscles [and each of the component, in the order of midpiece, 4th component, endpiece and 3rd component, were investigated in the same way as in the case of cations.

The results are shown in Tables XII, XIII, XIV and XV.

As seen in these tables, SO_4^{--} and $H_2PO_4^-$ interfered the combination of the midpiece, the endpiece and the 3rd component with sensitized corpuscles. However, on the combination of the 4th component with sensitized corpuscles, united with the midpiece previously, SO_4^{--} caused a remarkable inhibitory action, but HPO_4^{--} gave no effect.

SUMMARY

The effects of some cations and anions on the complementary action were investigated and following results were obtained.

Ca^{++}, Ba^{++}, Sr^{++}, Mg^{++}, SO_4^{--} and $H_2PO_4^-$ inhibited the action of the complement and by removal of them, the activity of the complement could be restored.

The mechaism of the anticomplementary action of these ions was based neither on the destruction of each factor necessary to the hemolytic reaction, such as hemolysins, red corpuscles or components of complement, nor on the effects upon the combination of the hemolysins and red corpuscles.

The inhibitory action of these ions was due to their interference on the combination of each component of the complement with sensitized

corpuscles. Exceptionally, only HPO_4^{--} did not inhibit the 4th component to combine with sensitized corpuscles.

These effects were shown to be caused only by the presence of the ions in the medium of the hemolytic system, but not by inactivation of complement due to the combination with ions. Even if the components due to the combination with ions. Even if the components of complement combine with them, the combination is probably reversible.

In conclusion the author wishes to express his hearty thanks to Prof. S. Fujimura for his kind guidance in this reserach.

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STUDIES ON THE N-TERMINAL LYSYLPEPTIDES OF α-CASEIN

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It has been demonstrated by Mellon *et al.* (*1*) that the N-terminal amino acids of α-casein are occupied with lysine and arginine. The present work was designed to determine the amino acid sequence in N-terminal lysine peptide of α-casein. By the procedures of Sanger's DNP method, several N-terminal di-DNP-lysylpeptides were separated from the ether extractable fractions of the partial hydrolyzates of DNP-α-casein and the amino acids present in these peptides were identified. From the results obtained a possible sequence of amino acid residues in N-terminal lysine peptide was discussed.

EXPERIMENTAL

Preparation of α-Casein—α-Casein was prepared from commercial casein according to the method of Warner (*2*). An electrophoretic study of the α-casein in phosphate buffer of pH 6.8 and 0.1 ionic strength, showed that it consisted of a single protein component.

Preparation of DNP-α-Casein—The dinitrophenylation was carried out by essentially the same way as described by Sanger (*3*). To the suspension containing 4.0 g. of α-casein, and 4.0 g. of sodium bicarbonate in 80 ml. of water, a solution of 4.0 ml. of dinitrofluorobenzene (DNFB) in 80 ml. of ethanol (95 per cent) was added gradually, the mixture was mechanically shaken for 3 hours and then allowed to stand over night at room temperature. The yellow precipitate of DNP-casein was centrifuged, washed with distilled water absolute alcohol, and anhydrous ether, and finally air-dried.

Hydrolytic Procedures—For partial hydrolysis three conditions were tried.

Experiment 1: DNP-α-casein was added to 100 volumes (*v./w.*) of (1:1) hydrochloric acid and the mixture was boiled under reflux for 10 hours. The timing was begun when the solution started to boil.

Experiment 2: DNP-α-casein was heated with 50 volumes (*v./w.*) of concentrated hydrochloric acid at 37° for 8 days in a sealed tube.

Experiment 3: DNP-α-casein was heated with 50 volumes (*v./w.*) of concentrated hydrochloric acid at 37° for 18 days in a sealed tube.

For complete hydrolysis the partial hydrolyzates were heated with (1:1) hydrochloric acid at 110° for 24 hours in sealed tubes.

Identification of the N-Terminal Amino Acids—DNP-α-casein was completely hydrolyzed with 100 volumes (*v/w*) of (1:1) hydrochloric acid in a sealed tube. After cooling the hydrolyzates were extracted three times with equal volume of ether.

The ether extracts were combined, washed with water, and taken to dryness *in vacuo*. The residues were subjected to the paper chromatography with water saturated *n*-butanol as solvent system to determine the N-terminal DNP-amino acids (*4*). In order to identify the parent amino acids, furthermore, the DNP-amino acids were heated for 8 hours in a sealed tube with 28 per cent ammonium hydroxide and the liberated amino acids were analyzed on one-dimentional paper chromatography with AcOH-BuOH-H_2O(1:4:5) as solvent system.

The acidic aqueous portions were subjected to talc adsorption chromatography described by Sanger (*5*) to separate DNP-amino acids from free amino acids and peptides included in the solution. The yellow band adsorbed at the top of the talc column was eluted with ethanol. After evaporating to dryness *in vacuo* the eluates were analyzed on the paper chromatography in usual manner.

Extractive Procedure of the N-Terminal Lysylpeptides—Sanger used ether for the extraction of DNP-amino acids and ethylacetate for the extraction of DNP-peptides, respectively. But Schroeder (*6*) has demonstrated that ether extraction gives cleaner extraction and removes all di-DNP-lysine and di-peptides and most of the tri- and tetrapeptides from the hydrolyzates. Therefore, ether has been used as a extracting solvent. The partial hydrolyzates were extracted three or four times with equal volume of ether. The combined extracts were washed with the equal volume of water to remove traces of free amino acids and peptides.

Removing Procedure of Dinitrophenol—The ether extracts of the hydrolyzates were concentrated *in vacuo* below 35°. The last traces of water remained were removed by adding a small quantity of acetone and reevaporating carefully under reduced pressure without splashing. Though the residues contained usually 2,4-dinitrophenol in a considerable amount, most of it could be conveniently removed by the Mill's cold finger sublimation technique (*7*).

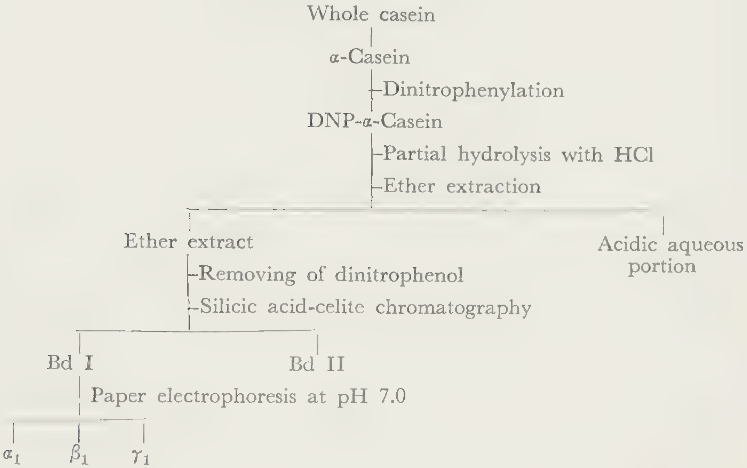
Separation of Di-DNP-lysylpeptides—The separation procedures are summarized diagrammatically in Table I.

1) *Separation by Adsorption Chromatography on Silicic Acid-Celite:* For the separation of DNP-amino acids the chromatographic techniques reported by Green and Kay (*8, 9*) were employed. Silicic acid (Merk) and celite 545, which were passed through a 60 mesh respectively, were throughly mixed in the portion of (4:1) (*w/w*). All the chromatography were carried out in the glass tubes of 20 mm. in inside diameter and 280 mm. in length ; the columns of adsorbent were 17±5 mm. in height.

The column was packed by dry packing method, under reduced pressure by applying full suction of an aspirator and it was prewashed by successive passing through the column with 0.2 V ml. of ether, V ml. of (1:1) acetone-ether, 0.8 V ml. of ether, V ml. of ligroin and V ml. of the developing solvent to remove essentially all the free water.

TABLE I

Separation of Di-DNP-lysylpeptides



The abbreviation V ml. mentioned above, is a volume of solvent required to wet completely a column of adsorbent. As a developing solvent 5 per cent acetic acid in benzene was used.

The sample (1.5 ml.) containing the ether extract of the partial hydrolyzate of 500 mg. DNP-α-casein was placed on the column. When the last of the sample solution passed into the adsorbent, the wall of the tube was washed with small amounts of the developing solvent. The adsorbent was extruded from the column and two yellow bands were cut and eluted with a (1:4) (*v./v.*) mixture of ethanol and ether. The solvent was then evaporated from the eluates under vacuum below 40°.

2) *Separation by Paer Electrophoresis:* The residues obtained above were subjected to electrophoresis on filter paper (Toyo No. 2) 14.8×40.0 cm. with 0.33 *M* phosphate buffer of pH 7.0. A potential of 400 volt, giving a current of 15 mA. was applied for 3 to 5 hours. The paper was dried at room temperature, then the yellow bands were cut out of the chromatogram and eluted with water.

Identification of Amino Acids Present in Peptides—The peptides isolated were hydrolyzed completely in sealed tubes with (1:1) hydrochloric acid and excess hydrochloric acid was removed *in vacuo*. The resulting amino acids were identified both by paper chromatography and by paper electrophoresis.

RESULTS AND DISCUSSION

N-Terminal Amino Acids—The paper chromatogram of the ether extractable compounds obtained from complete hydrolyzate of DNP-α-

casein gave two yellow spots (Rf: 0.70 and 0.55) which were found to be identical with di-DNP-lysine and 2,4-dinitrophenol, respectively. After heating with 28 per cent ammonium hydroxide, the parent amino acids of these yellow spots were found to be corresponding in Rf value to lysine. Mixed chromatogram gave only one spot with lysine. No α-DNP-arginine was given from DNP-amino acids present in the acidic portion of complete hydrolyzates both by chromatographic procedure and by Sakaguchi's raction. As Mellon (*1*) has discussed, it seems probable that the terminal arginines may be masked with a small amount of carbohydrate present in the commercial casein which was employed as starting material.

Separation of Di-DNP-lysylpeptides—Di-DNP-lysyl-peptides in the partial hydrolyzates were separated into several fractions as follows:

In the *Experiment 1*, Silicic acid—celite (4:1) column chromatogram gave two yellow bands, namely, Bd I (25 mm. from the top) and Bd II (130 mm. from the top). Further paper electrophoretic treatment for Bd I gave 3 bands (α_1, β_1, γ_1) and Bd II gave only one band. α_1 and β_1 travelled 2 cm. and 7 cm. from the reference mark towards the anode and γ_1 towards the cathode.

In the *Experiments 2 and 3*, paper electrophoretic procedure was carried out for 3.5 hours without silicic acid—celite column chromatography. *Experiment 2* gave 3 bands (α_2, β_2, γ_2) which travelled 3 cm. and 9 cm. towards the anode and 1.5 cm. towards the cathode, respectively. *Experiment 3* gav similarly three bands (α_3, β_3, γ_3) which travelled 2.3 cm. and 6.5 cm. towards the anode and 1.5 cm. towards the cathode, respectively.

Amino Acid Compositions of Di-DNP-lysylpeptides—The amino acid compositions of each peptides are shown in Table II.

Bd II gave Leu, Val, Ala, Glu, Asp and an unknown spot which developed between Val and Ala on the paper chromatogram with Ac-OH-*n*-BuOH-H_2O as solvent system. The re-hydrolysate gave Val, Glu and the same unknown spot. Therefore, Bd II seems to be a larger peptide containing Glu and Val, and having the strong resistance towards acid hydrolysis.

From the results obtained in Table I, it may be concluded that the amino acid sequence in the N-terminal peptide chain of α-casein is Lys-Leu-Val-Ala-Glu-Asp-.

TABLE II

Amino Acid Compositions of the N-Terminal Lysylpeptides of α-Casein

Hydrolytic condition	Peptide \ Amino acid	Di-DNP-Lys	Leu	Val	Ala	Glu	Asp
HCl (1:1) 10 hrs. reflux	α_1	+	++	++	++		
	β_1	+	++	++	++	+++	+
	γ_1	+	++	++			
Conc. HCl 8 days 37°.	α_2	+	++	++	++	+?	
	β_2	+	++	++	++	++	++
	γ_2	+	++				
Conc. HCl 18 days 37°.	α_3	+	++	++	++		
	β_3	+	++	++	++	++	
	γ_3	+	++	+?			

SUMMARY

1. The amino acid sequence in N-terminal lysylpeptides of α-casein has been investigated.
2. The di-DNP-lysylpeptides were separated by silicic acid—celite adsorption chromatography and by paper electrophoresis.
3. It was concluded that the N-terminal sequence is Lys-Leu-Val-Ala-Glu-Asp-.

The authors wish to express their gratitudes to Prof. T. Ando and Prof. H. Nishikawa for their guidance and encouragement and to Miss. R. Saeki for her technical assistance.

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STUDIES ON PROTEIN DENATURATION BY SURFACE CHEMICAL METHOD

II. ON THE MECHANISM OF SURFACE DENATURATION OF LYSOZYME

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In the previous paper (*1*), we have reported on the monolayer properties of lysozyme in relation to the urea denaturation in its spreading solution. It was found that the properties of the monolayers were profoundly affected by whether they were spread from the solution of native lysozyme or from the concentrated urea solutions in which the protein molecules were denatured. The surface pressure-area (F–A) curves of the films obtained from the latter solutions resembled the curves for ovalbumin and serum albumin etc. which have been so far investigated. However, the F–A curves of the films obtained from the acid solution of lysozyme had much smaller limiting area. It was suggested from these results that while the monolayers obtained from native lysozyme consist of the molecules of which forms are nearly the same as those in solutions, the molecules in the monolayers spread from the solutions of lysozyme denatured by urea are in a state of complete unfolding. We were, therefore, able to correlate the monolayer properties with the degree of urea denaturation of lysozyme in solutions by plotting the areas at constant pressure against the urea concentrations in the spreading solutions. Hence we may present the surface chemical method to investigate the protein denaturation in solutions. These facts show that the forms of protein molecules in solutions affect profoundly upon the monolayer properties ; unless some of the intra-molecular bonds which are essential to maintaining the native protein in its organized structure in solutions are destroyed before spreading, the completely unfolded films would not be readily obtained. One of the most important problems in the surface chemistry of proteins is to investigate the mechanism by which the globular protein molecules in solutions are unfolded when spread at an air-water interface. So far,

such an investigation has, however, scarcely been undertaken. The investigation described in this paper was an attempt to cast light upon the mechanism of surface denaturation in the case of lysozyme.

EXPERIMENTAL

Crystalline lysozyme used in this experiment was kindly provided by Prof. S. Akabori and Mr. K. Ohno.

Surface pressure was measured by the hanging plate method of Wilhelmy type. Aqueous solution of potassium carbonate in concentration of 0.01 *M* (pH 10.5) was used as substrate. To regulate the temperature of substrate, the glass hose was dipped in the trough, through which water at constant temperature was circulated from a thermostat (*2*). The spreading solutions were the same as those used in the previous experiment (*1*). Native lysozyme was spread from a solution in *N*/1000 hydrochloric acid containing 2 per cent isopropyl alcohol. Alkali denatured lysozyme was prepared by dissolving the protein in sodium hydroxide solution in a given concentration and then 2 per cent isopropyl alcohol was added. In the case of denatured lysozyme by urea 40 per cent of isopropyl alcohol was added to the solution prior to spreading after dissolution was complete in solution of a given concentration of urea.

RESULTS AND DISCUSSION

When native lysozyme was spread from a solution in *N*/1000 hydrochloric acid containing 2 per cent isopropyl alcohol, the F–A curves obtained were profoundly affected by the temperature of the substrate (Fig. 1). The higher was the temperature of the substrate, the larger became the areas at which surface pressures were steeply increased.

The effect of the temperature of the substrate on the F–A curves of the monolayers which were spread from the urea solution (8 *M*) of lysozyme is shown in Fig. 2. In this case the effect of temperature was also the same as that shown in Fig. 1. All the curves in Figs. 1 and 2 were obtained for the monolayers which were allowed to stand on the surface for 30 minutes before measurements.

In Fig. 3 the areas at constant pressure (2 dynes/cm.), which were obtained from the F–A curves shown in Figs. 1 and 2, are plotted against the temperatures of substrate.

When native lysozyme was spread (Fig. 3, Curve I), the area was suddenly increased at about 15°. It is expected that the area will approach to a maximum constant value (Curve III) at about 35°. In the case of urea denatured lysozyme (Curve II) the area was increased at about 9° and became constant at 18°. In this figure, the

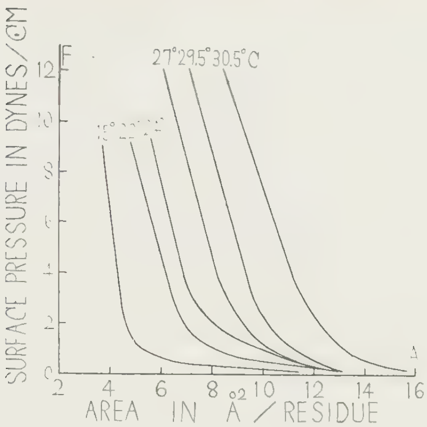

Fig. 1. The effect of temperature of substrate on the F–A curves of lysozyme monolayers.

Spreading solution : N/1000 hydrochloric acid.

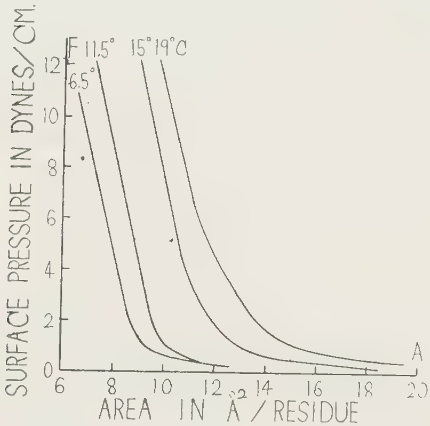

Fig. 2. The effect of temperature of substrate on the F–A curves of lysozyme monolayers.

Spreading solution : 8 M urea solution.

maximum constant area, the straight line parallel to the temperature axis (Curve III), represents the upper limit of expansion, beyond which the area never exceeds even if lysozyme is spread from the solution in which lysozyme molecules are considered to be denatured to the greatest extent, that is, this area (13.2 Å^2/residue at 2 dynes/cm.) would be the

maximum expansion of lysozyme which might be completely unfolded at interface. For instance, heat treatment of lysozyme in 8 *M* solution of urea in a thermostat at 55° for two hours led to the area on Curve III on the substrate at 11° and the area obtained from the solution in 10 *M* urea was also found on this curve.

The areas at 2 dynes/cm. which were obtained for the F–A curves of the films spread from the various solutions are also shown in Fig. 3. It is interesting to note that the points shown in this figure are grouped into three Curves I, II and III.

The first group is represented by Curve I, which was obtained for the films spread from *N*/1000 hydrochloric acid solution. The films spread from 5 *M* urea solution and from *N*/1000 hydrochloric acid solution which was heated to 65°±3° for two hours beforehand, occupied the areas on Curve I. This fact shows that lysozyme molecules are not denatured in these solutions. It is generally accepted that lysozyme in acid solution resists against heat and that it is difficult to be denatured in urea solutions (*3*).

The second group is represented by Curve II, which was obtained for the films spread from 8 *M* urea solution. The films spread from the fresh solutions in *N*/50 and *N*/25 sodium hydroxide containing 2 per cent isopropyl alcohol belong to this group. The films spread from the old solutions in *N*/50 sodium hydroxide and *N*/1000 hydrochloric acid which were preserved in a kelvinator at about 0° for more than a month, also belong to this group. This shows that lysozyme is denatured in these solutions and corresponds to the fact that lysozyme molecules are unstable in alkaline solutions (*3*). All the areas occupied by the films spread from the solutions in which lysozyme molecules are considered to be denatured in some extent accumulated on Curve II and did not depend on their denaturing agents. This fact suggests that there may be some definite bonds which cause the fatal destruction of the highly organized structure of lysozyme molecule by their breaking. The bonds broken might be independent of the kind of denaturant.

The third group corresponds to Curve III. This is the case for the monolayers of lysozyme which suffered most drastic denaturation in solutions, as described above. Furthermore, the areas occupied by the films spread on the substrates of which temperatures were sufficiently high fall on Curve III, irrespective of its spreading solution.

The phenomenon described above should be designated as "surface heat denaturation" of lysozyme. Next, the cause of this phenomenon will be considered.

Cheesman and Schuller (*4*) examined the surface inactivation of pepsin and found that the films spread from its aqueous solution had much smaller area than that from the solution in 60 per cent isopropyl alcohol and that the former film was found to be active enzymatically, whereas the latter was inactive. Furthermore, they assumed that " the structure of the films of difficultly spread proteins such as myosin, is such that it consists of an upper layer of orientated polypeptide chains and bears on its lower surface the adsorbed layer of unaltered or partially altered molecules which contribute materially neither to the surface pressure nor to the film potential ".

In order to analyze our results on lysozyme films we make the same assumption as Cheesman and Schuller did. That is, the area represented by Curve III in Fig. 3 corresponds to that of the films which consist of the completely unfolded molecules of lysozyme and in the case of smaller expansion than this area, the films consist of two different types of molecular configurations, one of which is in a state of complete surface denaturation and the other, globular or partially altered configuration. The latter configuration is assumed by the molecules existed in the adsorbed layer and does not contribute to the surface pressure. However, it is not clear whether the " globular " molecules existed in the adsorbed layer would have the same configuration as that in solutions or not.

By these assumptions, C_n in Eq. (1) represents the concentration of the " globular " molecules in adsorbed layer.

$$\frac{1}{A} - \frac{1}{A_d} = C_n \qquad (1)$$

where A is the area at any given expansion and A_d the area represented by Curve III in Fig. 3. The globular molecules existed in adsorbed layer gradually suffer surface denaturation with time giving rise to completely unfolded films. Because the curves shown in Fig. 3 were obtained for the films all of which were stood for 30 minutes before measurements, the relations indicate that the amounts of reactant suffered surface denaturation are different by the temperature of substrate.

Therefore, the rate constant of surface denaturation must be obtained if the F-A curves of the films are measured on the substrate at constant temperature at different ages after spreading.

The effect of the age upon the F–A curves are shown in Fig. 4. It si found from this figure that the F–A curves developed to larger areas with time. The logarithms of the concentrations of " globular "

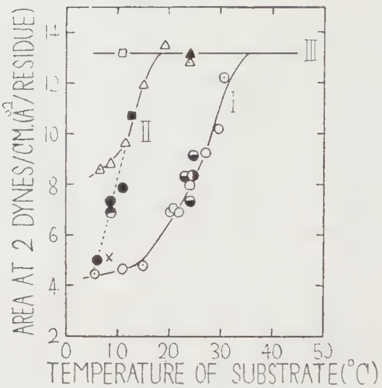

Fig. 3. The relationships between the areas at 2 dynes/cm. and the temperatures of substrate.

Spreading solutions: ⊙, $N/1000$ hydrochloric acid (fresh); ⊖, 5 moles urea solution; ◑, $N/1000$ hydrochloric acid, heated at 65°±3° for two hours prior to spreading; △, 8 M urea solution; ⊡, 8 M urea solution heated at 55° for two hours prior to spreading; ▲, 10 M urea solution; ■, $N/50$ sodium hydroxide (old); ●, $N/1000$ hydrochloric acid (old); ◓, $N/25$ sodium hydroxide; ×, $N/50$ sodium hydroxide (fresh).

molecules in adsorbed layer, C_n, which are calculated from Eq. (1) by using the areas at 2 dynes/cm., are plotted against time in Fig. 5 and linear relations were obtained. This is the indication that the surface denaturation obeys the first order kinetics; hence Eq. (2) should be applicable:

$$k = \frac{1}{t} \ln \frac{C_0}{C_n} \qquad (2)$$

where k is the rate constant, t the elapsed time and C_0 the initial concentration.

The rate constants, k, at 11° and 21°C, which were obtained from Fig. 5 were 3.7×10^{-5} and 9.0×10^{-5} sec.$^{-1}$, respectively.

By means of Arrhenius equation,

$$k = Ae^{-E/RT} \qquad (3)$$

the value of activation energy, E, might be calculated to be 15 kcal./mole.

Gorni and Felix (*5*) examined the heat inactivation of lysozyme in solutions and found that the process was of the first order and that the activation energy for this process was 42 kcal./mole. This data

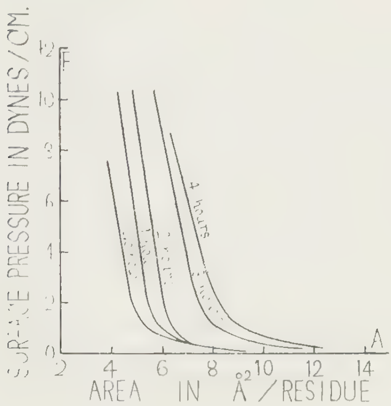

FIG. 4. The effect of age on the F-A curves. The temperature of substrate : 11°.

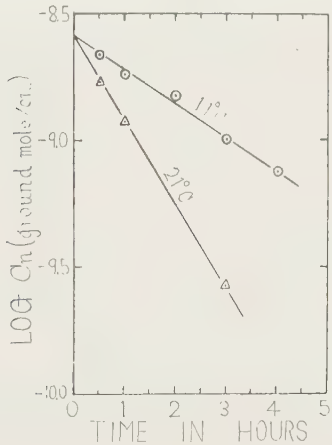

FIG. 5. The relationship between log C_n and the elapsed time.

in bulk is about three times as great as the value of 15 kcal./mole in the present case of surface denaturation. This is caused by either or both of the following two reasons, one of which is that an air-water interface would catalyze the reaction of the heat denaturation and play a part for reducing the activation energy, and the other is that the configuration of the "globular" molecules which have been assumed to exist

in the adsorbed layer would be considerably different from the native state in solutions and would be rather labile.

From the above considerations, it is found that the activation energy of 15 kcal./mole is necessary in order to obtain the monolayers consisting of completely unfolded molecules when native lysozyme is spread at an air-water interface. This is the reason why the F–A curves of lysozyme films are profoundly affected by the temperature of the substrate, as shown in Fig. 1. When the proteins such as ovalbumin, serum albumin, *etc.* which have been so far investigated in detail, are spread as monolayers, the activation energy to pass from globular molecules to completely unfolded ones would be very low and then the completely unfolded films are formed instantaneously. Therefore, in the case of these protein films, neither the effect of temperature nor the time effect have been observed. However, the effect of aging before the start of compression after the spreading of the film was observed for the films of some proteins such as insulin and pepsin (*6*) and this fact may also be interpreted by the mechanism as in the case of lysozyme. It would be assumed that lysozyme molecules have very rigid structure in solutions and are not so readily denatured by surface as other proteins such as ovalbumin.

The similar effect of temperature of the substrate on lysozyme films spread from native form was also observed with the films spread from the solution containing urea in concentration of 8 *M* (Curve II in Fig. 3), although the curve passed to lower temperature region. This fact suggests that lysozyme molecules in this solution would not be completely denatured by urea and that their structure are not so labile to suffer surface denaturation and to spread completely unfolded films instantaneously. To denature the protein molecules in spreading solutions beforehand has the effect of reducing the activation energy to pass into the completely unfolded films.

Katchalsky and Miller (*7*) have found that the adsorption layer of polymethacrylic acid did not show any tendency to pass into a monolayer and that the monolayers of polymethacrylic acid could not spread even after very long time intervals. They interpreted this fact as follows: a sufficiently high potential barrier separartes the two molecular forms to prevent a thermal passage from hyper-coiled state in adsorbed layer to the film state. The hypercoiling is due to strong intra-molecular hydrogen bonding of the carboxyl groups. In order to pass from the adsorbed layer to the monolayer the molecules should first open up and expend the work necessary to break the hydrogen bonds. From these considerations they calculated the height of potential barrier which separates

the adsorbed state and the film state. Such a consideration corresponds to our interpretation on lysozyme films.

According to Eyring and Stearn (*8*), free energy (ΔF^*), heat (ΔH^*) and entropy (ΔS^*) of activation can be estimated by the following three equations:

$$k = \frac{\mathrm{k}T}{h} \mathrm{e}^{-\Delta F^*/RT} \quad (4)$$

$$\Delta H^* = E - RT \quad (5)$$

$$\Delta S^* = \frac{\Delta H^* - \Delta F^*}{T} \quad (6)$$

and are shown in Table I.

TABLE I

Surface Denaturation of Lysozyme
(at 11°C)

ΔF^* (*kcal./mole*)	ΔH^* (*kcal./mole*)	ΔS^* (*cal./deg./mole*)
22.4	14.4	−28

With protein denaturation, in general, there are large increases in entropy and heat during the activation process. In this case, however, there is a decrease in entropy and a small increase in heat. The reason for this is not clear. Whether or not the globular molecules which are assumed to exist in adsorbed layer would have the same configuration as that in solution is not clear. So the activation data cannot be compared directly with the data of usual denaturation in bulk. There are two possibilities to explain the negative value of entropy: (a) that involved in a decrease in degree of freedom at interfaces compared with that in solutions and (b) that involved in freezing water molecules to the protein molecules.

SUMMARY

In order to clarify the mechanism of surface denaturation of lysozyme, the effects of the temperature of substrate and of the elapsed time before measurements on the F–A curves were studied. The results obtained were as follows:

(1) By plotting the areas at constant pressure against the temperatures of substrate, the points were found to be grouped into three curves,

one of which was obtained from the solution in which lysozyme molecules were not denatured. The second, the curve was obtained from the solutions in which the protein molecules were denatured and the accumulation of the points around this curve was independent of the denaturing agents. The third, the curve was obtained from the solutions in which the denaturation was rather drastic and on the water surface of which temperatures were sufficiently high.

(2) When native lysozyme molecules were spread on water surface, the surface denaturation was of a first order process. The activation of 15 kcal./mole was necessary for the surface denaturation of the globular molecules in adsorbed layer. This is the reason why the F–A curves of lysozyme were profoundly affected by the temperature of substrate and by the aging before measurements. It was suggested that this activation energy for the proteins which had been so far investiggated would be so small that the effects of temperature were not observved, and that to denature proteins in solutions beforehand plays a role of reducing this activation energy.

In conclusion, the author expresses his hearty thanks to Prof. T. Isemura for his kind guidance throughout the present work, and to Prof. S. Akabori and Mr. K. Ohno in Faculty of Science, Osaka University who kindly supplied the valuable sample to him.

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THE ENZYMATIC PHOSPHORYLATION OF SEDO-HEPTULOSE*

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(Received for publication, July 19, 1955)

In a previous paper (*1*) we reported that a *Bacillus* species, Strain W–2, actively utilizes sedoheptulose when adapted to this sugar. This utilization was found to occur only in the presence of both inorganic phosphate (or ATP*** when dried cells were used) and Mg^{++}. This finding together with an examination of the effects of certain inhibitors strongly suggested the possibility that the initial step of the ultilization process may be the formation of a sedoheptulose phosphate.

The present studies with a cell-free extract from the heptose-adapted cells of Strain W–2 have now clearly disclosed the existence of a phosphokinase which catalyzes the phosphorylation of sedoheptulose with ATP in the presence of Mg^{++} (or Mn^{++}). It is the purpose of this communication to report the properties of this phosphokinase and to present evidence that the phosphorylation product is a sedoheptulose monophosphate.

EXPERIMENTAL

Materials—The sedoheptulose preparation used in the previous report (*1*) was further purified by deionizing through two columns of ion exchange resins, *i.e.*, Amberlite IR-120, H^+-form, and IRA-410, OH^--form. The deionized solution was evaporated *in vacuo* to a thick syrup below 60°. The sedoheptulose content of this purified syrup was found to be 96 per cent when analyzed by the method desacribed below. ATP (Ba salt) was prepared from rabbit muscle by the method of Szent-Gyorgyi (*2*) and used after converting it to Na salt.

Preparation of Cell-free Extract—Sedoheptulose-adapted cells of Strain W-2 were obtained and washed as described previously (*1*). The washed cells were mixed with 5 parts of powdered glass and ground in a chilled mortar for 15 minutes, treated with

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** SHOKI ARAI.

*** ATP=adenosine triphosphate, ADP=adenosine diphosphate.

an appropriate amount of cold 0.1 *M* bicarbonate buffer, pH 9.4.* and the glass powder and the cell *dèbris* were removed by centrifugation. The bicarbonate extract was brought to 50 per cent saturation with respect to ammonium sulfate by the addition of saturated ammonium sulfate solution. The precipitate was collected by centrifugation and again dissolved in a bicarbonate buffer of the same pH. The ammonium sulfate precipitation was repeated once more as above and the precipitate was finally dissolved in distilled water. This solution containing 5–15 mg. of solids per ml. was used as cell-free extract.

Analytical Procedures—Except where otherwise noted, deproteinization was carried out by adding 0.2 volume of 20 per cent trichloroacetic acid to the reaction mixtures and removing the precipitate by centrifugation.

Acid-labile (hydrolyzable in 7 minutes in 1 *N* HCl at 100°) phosphate was estimated according to the directions of Umbreit (*4*) except that the determination of inorganic phosphate was carried out by the method of Nakamura (*5*).

The procedure of sedoheptulose determination was based on the principle described by Klevstrand and Nordal (*6*). Three millilitres of Drury's orcinol reagent (*7*) were added to 1 ml. of deproteinized sample** and heated in a boiling water bath for 40 minutes, cooled rapidly by exposing the test tube to running tap water, and diluted to 10 ml. with 85 per cent ethanol. The blue color thus developed was measured in a Hitachi photoelectric colorimeter (Model A) using the " R " filter. Crystalline dibenzalsedoheptulosan (dibenzalanhydrosedoheptulose), prepared by the method of La Forge and Hudson (*8*), was used as standard material for the colorimetry, since this compound exhibits the same extinction coefficient with that of sedoheptulose in the orcinol test (*6*). This method is capable of determining sedoheptulose in 1 ml. of sample containing $0.5–11 \times 10^{-5}$ mole/lit. of the heptose.

The phosphate ester of sedoheptulose, which is the product of the phosphokinase reaction, also reacts with orcinol reagent to give a blue color. It was, therefore, necessary to remove the ester prior to the estimation of the free heptose. This could be done as follows by taking advantage of the fact that barium salt of the heptose phosphate as well as those of adenine nucleotides is insoluble in ethanol in which the free sugar remains soluble. To 3 ml. of deproteinized sample were added 0.2 ml. of 25 per cent barium acetate solution and the pH was brought to 8.2 with KOH. The mixture was then diluted to 30 ml. with ice-cold 95 per cent ethanol, the insoluble barium salts were removed by centrifugation, and the supernatant fluid was used for free sedoheptulose

* Lampen (*3*) showed that 0.1 *M* bicarbonate buffer of pH 9.4 is effective in destroying the enzyme system concerned in further metabolism of pentose phosphates in extracts of pentose-adapted bacteria without affecting the activity of pentokinases.

** In the previous paper (*1*), where intact cells were used, trichloacetic acid was not suitable as deproteinizing agent for sedoheptulose determination possibly due to the extraction of Bial-sensitive substances from the cells. The same reagent was, however, found to be quite satisfactory in the present investigation using only cell-free preparations.

determination.

Paper chromatography was carried out by the ascending method using Toyo Roshi No. 50 paper. Spots of phosphate compounds were developed by spraying the $FeCl_2$-sulfosalicylic acid reagent of Wade and Morgan (*9*). The orcinol-trichloroacetic acid reagent of Bevenue and Williams (*10*) was employed for detecting sedoheptulose spots.

Isolation of Crude Reaction Products—A mixture of 5 ml. of the cell-free extract (dry weight 251 mg.), 6 ml. of 10^{-3} *M* sedoheptulose in 0.1 *M* citrate buffer of pH 6.4, 5 ml. of 10^{-3} *M* ATP, and 6.5 ml. of 4×10^{-2} *M* $MgCl_2$ was incubated for 3 hours at 35°. The reaction was stopped by adding 4 ml. of 20 per cent trichloroacetic acid, the protein precipitate was centrifuged off, and the clear supernatant fluid was subjected to fractionation according to the procedure of Le Page (*4*) for phosphate compounds. The alcohol-insoluble fraction containing barium salts of sedoheptulose phoaphate and adenine nucleotides was collected and dried with a large amount of acetone. The yield of this crude mixture of barium salts was 15 mg. This was converted to free phosphates by the usual method prior to chromatographic analysis.

Hydrolysis of Products by Phosphatase—12 mg. of the crude reaction products (alcohol-insoluble, barium-soluble) obtained by further fractionation were converted to sodium salts and dissolved in 4 ml. of 0.1 *M* citrate buffer of pH 6.0. To this were added 20 mg. of a phosphatase preparation in 2 ml. of distilled water and the mixture was incubated for 60 minutes at 30°. The mixture was then deproteinized with 1 ml. of 20 per cent mercuric acetate and the filtrate was applied to paper chromatography. The phosphatase preparation used was obtained from sweet potato by following the procedure of Watanabe *et al.* (*11*) to the first ammonium sulfate precipitation and the precipitate was dried with acetone.

Assay for Phosphokinase Activity—Routine assay for the phosphokinase activity was carried out by determining the decrease of acid-labile phosphate from the reaction mixture which contained each appropriate amount of sedoheptulose, ATP, Mg^{++}, cell-free extract, and buffer and was made up to 6 ml. If necessary, other additions or omissions were made, but the total volume was always kept to 6 ml. The reaction was performed in an open tube at 35° and the incubation was usually continued for 60minutes.

RESULTS

Demonstration of Phosphokinase Activity in Cell-free Extract—When the cell-free extract from the sedoheptulose-adapted cells of Strain W-2 was incubated with ATP and sedoheptulose in the presence of Mg^{++}, a considerable decrease of acid-labile phosphate was observed. Typical data of such experiments are presented in Fig. 1. It will be seen in the figure that only a small amount of acid-labile phosphate is lost if sedoheptulose is omitted from the complete system. This fact excludes the possibility that ATP-ase or apyrase is responsible for the loss of acid-labile phosphate

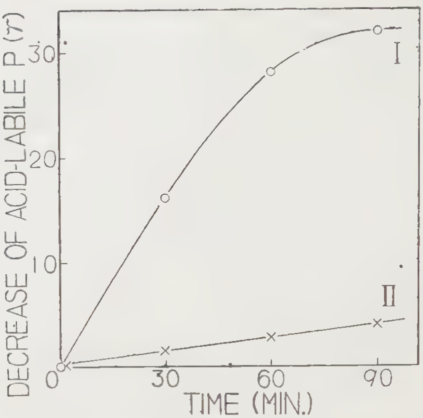

FIG. 1. Activity of a phosphokinase in an extract of Strain W-2.

I : Complete system consisted of enzyme solution (dry weight 3.2 mg.), 0.002 *M* sedoheptulose, 0.002 *M* ATP, 0.01 *M* $MgCl_2$ and 0.05 *M* citrate buffer in a final volume of 6 ml. Incubation was carried out aerobically at 35°.

II : Control system, the same except the sedoheptulose was omitted

in the presence of sedoheptulose.

It is more likely to attribute the decrease in the complete system to the transfer of the terminal labile phosphate of ATP to the heptose molecule. It was, in fact, confirmed that a simultaneous disappearance of the free heptose takes place along with the decrease of acid-labile phosphate as illustrated in Fig. 2.

Fig. 2 also indicates that the loss of each mole of acid-labile phosphate is accompanied by the disappearance of 1.1–1.2 moles of the free sugar. It was, therefore, suggested that an energy-rich phosphate group of ATP is transferred to the heptose under the influence of a phosphokinase to form a sedoheptulose monophosphate.

Chromatographic Evidence for Phosphokinase Activity—In order to confirm the phosphorylation of sedoheptulose, the crude mixture of reaction products obtained as described above was analyzed by paper chromatography. As will be seen in Table I, three distinct spots of phosphorous compounds were observed with a solvent mixture of *n*-butanol, water, and acetic acid (4:2:1). Among them one which satyed on the starting point seems to be ATP. The other two spots have not yet been identified,

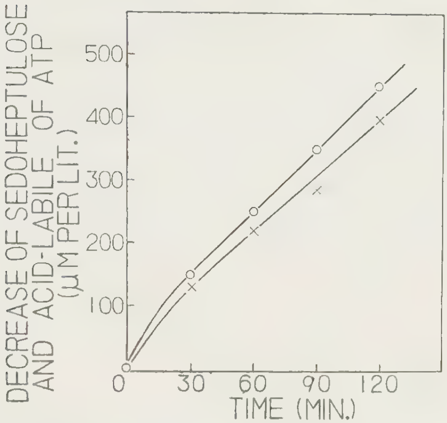

FIG. 2. Simultaneous disappearance of free sedoheptulose and acid-labile P.

I : Decrease of free sedoheptulose.

II : Decrease of acid-labile P.

Reaction mixture contained 1 ml. of enzyme solution (dry weight 3.0 mg.), 0.002 *M* sedoheptulose, 0.002 *M* ATP, 0.01 *M* $MgCl_2$ and 0.05 *M* citrate buffer (pH 6.4) in a final volume of 6 ml. Incubation was carried out at 35°.

TABLE I

Paper Chromatography of Reaction Products

Samples	Spraying reagents	Spots (Rf-values)			
Crude reaction products	Fe-Sulfo**	0.0	0.16	0.22	
,, (after fractionation and hydrolysis*)	Orcinol***				0.34
Sedoheptulose	Orcinol				0.34
ATP	Fe-sulso	0.0			

* Hydrolysed by a phosphatase preparation (see the text).

** $FeCl_3$-sulfosalicylic acid reagent specific for phosphorous compounds.

*** Orcinol-trichloroacetic acid reagent specific for ketoheptoses.

but it appears probable that they represent sedoheptulose phosphate and ADP, respectively. Decisive evidence that the products contain a sedoheptulose phosphate was, however, provided by the finding that they

give a spot corresponding to sedoheptulose after hydrolysis by a phosphatase preparation. No such spot was observed in the chromatogram of the unhydrolysed products.

Effect of pH—The effect of pH on the activity of the phosphokinase was studied by measuring the rate of the decrease of acid-labile phosphate. Citrate buffer was used in the pH range from 5 to 7 and borate buffer for 7 to 10. As is illustrated in Fig. 3, the pH-activity curve showed two maxima at about pH 6.4 and at 9.0. This two-peaked curve was confirmed in repeated experiments.

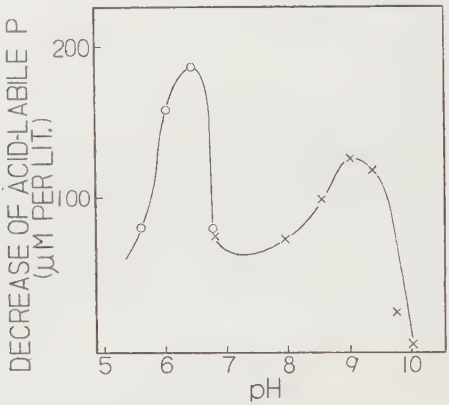

FIG. 3. Effect of pH

○ ; 0.022 *M* citrate buffer. × ; 0.022 *M* borate buffer.

Reaction mixture contained enzyme solution (dry weight 1.2 mg.), 0.007 *M* sedoheptulose, 0.007 *M* ATP, 0.02 *M* $MgCl_2$ and citrate or borate buffer solution. Incubation was carried out for 60 mins. at 35°.

Metal Requirement—Mg^{++} is indispensable for the kinase activity. The effect of Mg^{++} concentration of the activity was studied and the results are shown in Fig. 4. It will be seen that the optimum concentration of Mg^{++} is as high as 0.01 *M* under the conditions employed.

Both $MgCl_2$ and $MgSO_4$ exhibited the same effect. Among other metals tested, only Mn^{++} was found to be able to replace Mg^{++} as activator. But its efficiency was much lower than that of Mg^{++} at the same concentration (Fig. 5).

Effect of Inhibitors—Table II records the data on the effects of several inhibitors on the phosphokinase activity. Considerable inhibitions by

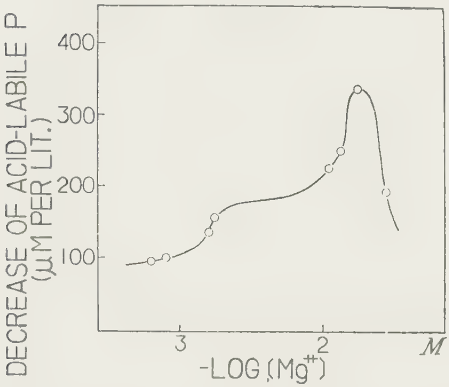

FIG. 4. Effect of Mg++ concentrations on decrease of acid-labile P.
Reaction mixture consisted of enzyme solution, 0.002 *M* sedoheptulose, 0.002 *M* ATP, 0.05 *M* citrate buffer (pH 6.4) and $MgCl_2$ solution. Incubation was carried out for 60 minutes at 40°.

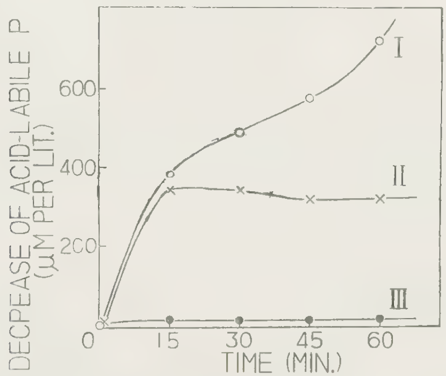

FIG. 5. Effects of Mg++ and Mn++
I : Mg++ experiment, 0.015 *M* $MgCl_2$. II : Mn++ experiment, 0.015 *M* $MgCl_2$. III : Control experiment (without metals)
All other conditions were the same as in Fig. 4.

iodoacetate and copper salt suggested the participation of SH-groups in the activity. This was more clearly shown in experiments where the inhibitory action of alloxane was measured in the presence and absence of cysteine. As is shown in Fig. 6, alloxane inhibits the phosphokinase reaction at concentrations higher than 0.001 *M*. This inhibition was

TABLE II

Inhibitions of Phosphokinase

Inhibitor	Concentrations	Inhibition
	M	*per cent*
NaF	0.002	28
	0.001	11
NaN_3	0.01	80
	0.001	72
Iodoacetate	0.01	60
	0.002	35
$CuSO_4$	0.01	95
	0.001	70

The conditions were the same as Fig. 3 except the pH was kept at 6.4 with citrate buffer and inhibitors were added to final concentrations indicated.

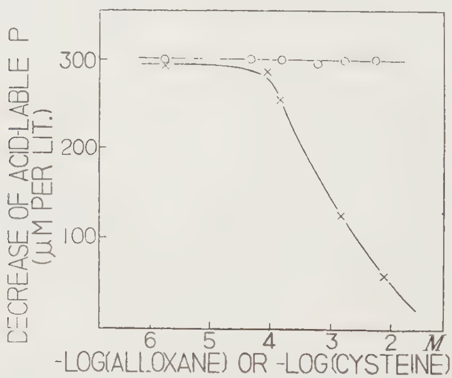

FIG. 6. Inhibition by alloxane and its restoration by cysteine.

I : Alloxane experiment—Reaction mixture consisted of enzyme solution, 0.002 *M* sedoheptulose, 0.002 *M* ATP, 0.01 *M* $MgCl_2$, 0.05 *M* citrate buffer (pH 6.4), 0.5 ml. of alloxane solution and 0.5 ml. of water in a final volume 6.5 ml.

II : Cysteine restoration—In this restoration experiment water was replaced by cysteine solution. The enzyme solution was pre-incubated with cysteine solution for 30 minutes, and substrates were added. Incubation was carried out for 60 minutes at 35°.

found to be restored if cysteine was added at the same concentration as that of alloxane.

Specificity—As is shown in Table III, the cell-free extract possessed little or no phosphokinase activity to several hexoses and pentoses. It was also found that the presence of gulcose, fructose, and arabinose does not affect the kinase activity to sedoheptulose at the same concentratis to that of the substrate. This indicates that the kinase has no affinity to these hexoses and pentoses.

TABLE III

Substrate Specificity of Phosphokinase

Sugars	Decrease of acid-labile P
	μM per lit.
Sedoheptulose	402
Glucose	10
Fructose	65
Galactose	0
Arabinose	50
Xylose	25
None	20

The conditions were the same as Fig. 3 except that pH was 6.3 (citrate buffer 0.02 *M*), dry weight of extract per vessel was 9 mg., and sugars indicated were added to each vessel at the final concentration of 10^{-3} *M*.

DISCUSSION

The evidence reported in this paper indicates that a phosphokinase catalyzing the reaction.

Sedoheptulose + ATP → sedoheptulose monophosphate + ADP,

can bep roduced adaptively in the cells of Strain W-2 when they are grown in the presence of sedoheptulose. This kinase is not capable of acting on several common hexoses and pentoses. It seems, therefore, that this enzyme is different from any other hexo- and pentokinases hitherto described. We are not able, however, to decide if it is specific to sedoheptulose until the specificity test is extended to a wider group of substances including other heptoses than sedoheptulose. Several properties of this enzyme such as its requirement of Mg^{++} or Mn^{++} and inhibition by fluoride or SH reagents are quite common to a number of phosphokinases

(see, *e.g.*, (*12*)). The finding that Mn^{++} activates the cell-free kinase appears to be inconsistent with the inhibitory action of this metal upon the sedoheptulose utilization by dried cells (*1*). This may, however, be explained if one assumes that an enzyme or enzymes sensitive to this metal might be involved in the further metabolism of the heptose phosphate.

That the product of the phosphokinase reaction is a sedoheptulose monophosphate is suggested by the mole-to-mole stoichiometry between the disappearance of the free heptose and the decrease of acid-labile phosphate. It is also indicated that isomerization or other alterations of the heptose molecule does not occur during the phosphorylation process, since sedoheptulose can be recovered on paper chromatogram of the phosphatase-hydrolyzed reaction products. Although further investigations are needed to clarify the position of phosphate group in the heptose phosphate, we have some evidence to infer that the product may be sedoheptulose-7-phosphate. This inferrence has been obtained from an experiment which showed that the rate of acid hydrolysis of the product is of the same order as that of fructose-6-phosphate. It may also be reminded here that sedoheptulose appears in animal and plant metabolisms in the form of 7-phosphate (*13*, *14*, *15*).

The double-peaked pH-curve for the phosphokinase activity is in accord with the corresponding curve for the effect of pH on sedoheptulose utilization by intact cells where two maxima (at about pH 6 and 9) were also noticed (*1*). The reason of this phenomenon remains, however, to be elucidated.

SUMMARY

1. A phosphokinase catalyzing the phosphorylation of sedoheptulose with ATP was found in a cell-free extract of a *Bacillus* species, Strain W–2, grown in the presence of this sugar.

2. An equimolar relationship was observed between the disappearance of free heptose and the decrease of acid-labile phosphate in the phosphokinase reaction. This suggests that the reaction product is a sedoheptulose monophosphate.

3. The reaction products were isolated as crude barium salts which gave a spot corresponding to sedoheptulose on paper chromatogram after (but not before) hydrolysis by a phosphatase preparation.

4. The pH-activity curve of this enzyme has two maxima at pH 6.4 and 9.0.

5. The enzyme requires Mg^{++} or Mn^{++} for its activity, Mn^{++} being less effective as activator.

6. NaF, NaN_3, iodoacetate, Cu^{++}, and alloxane are inhibitory to the enzyme. The inhibition by alloxane can be reversed by cysteine.

7. Several common hexoses and pentoses are inactive to the enzyme. They do not interfere with the phosphorylation of sedoheptulose.

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PRESENCE OF SERO-LACTAMINIC ACID AND GLUCOSAMINE AS CONSTITUENTS OF SERUM MUCOPROTEIN

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Walz (*1*) and Levene and Landsteiner (*2*) were the first to report the presence of a material which gives purple color by heating with Bial's reagent. In the course of studies on lipoidosis, Klenk (*3*) noted the same substance in an infantile amaurotic idiocy brain, which afterwards was isolated in crystalline state and designated as 'neuraminic acid' (*4*).

On the other hand, Blix (*5*) obtained from submaxillary mucin a crystalline reducing polyhydroxylic acid corresponding to the formula, $C_{14}H_{24}NO_{11}$ which produces with Bial's orcinol reagent violet pigment soluble in amyl alcohol. The name 'sialic acid' was assigned later by Werner and Odin (*6*).

In 1951, Yamakawa and Suzuki isolated from hematoside, an equine blood stroma glycolipid, 'hemataminic acid' (*7*) and assumed it as a methoxyl derivative of 2-amino-desoxynonuronic acid or prehemataminic acid (*8*). The identity of it with neuraminic acid was subsequently confirmed by Klenk and Wolter (*9*). Methoxy-neuraminic acid or hemataminic acid was thereafter isolated from submaxillary mucin (*10*), human milk (*11*) and liver of amyloid degeneration (*12*).

'Lactaminic acid', found in bovine colostrum by Kuhn and Brossmer (*13*) was similarly Bial-positive. In their latest work, Klenk and Faillard (*14*) obtained *N*-acetyl-neuraminic acid besides methoxyl-neuraminic acid in the mucoprotein of submaxillary mucin.

All these substances possess the common characteristics of forming purple color not only with Bial's orcinol reagent but also with Ehrlich's aldehyde and Dische's diphenylamine reagents.

On the other hand, Niazi and State (*15*) demonstrated the increase of color intensity given by serum heated with diphenylamine reagent in the case of malignant diseases. Subsequent studies have

shown the color to be associated with serum mucoprotein; Werner and Odin (*6*) supposed it to be due to 'sialic acid'. Numerous studies involving the isolation and characterization of the chromogen have been carried out, but no one has succeeded.

The present authors have revealed that the chromogen can be split from serum mucoprotein by heating at pH about 1 to 2 for half an hour and purified by adsorbing on an anion exchanger, resulting finally in isolation in crystalline form as described in experimental part.

The nature of the product is very similar to lactaminic acid, but our substance is reducing, contrary to Kuhn and Brossmer's description; the term 'sero-lactaminic acid' is, therefore, tentatively assigned to it.*

The presence of hexosamine as a component of serum glycoproteins was established very early. Odin and Werner (*17*) reported that both glucosamine and chondrosamine appear to be present. Our present study with human, equine and rabbit sera as well as purified human orosomucoid (Winzler's acid glycoprotein) indicated absence of chondrosamine therein, because by Gardell's chromatographic technique (*18*) only one peak corresponding to glucosamine was observed. Whereas the proportion of glucosamine to chondrosamine was widely variable for glycolipid of red blood cells of various species of animals, no similar relationship was found with serum hexosamine.

Since it has been shown that the amounts of lipid-hexosamine and lipid-hemataminic acid were significantly different from each other in various animal erythrocytes (*19*), the estimation of both contents was carried out with various animal sera in order to determine whether the sero-lactaminic acid content runs parallel to the amount of glucosamine. The concentrations of both components distribute somewhat characteristically with species and no significant deviation was found in normal sera.

A great number of studies, particularly during the past few years, have indicated that the concentration of the serum glycoproteins may be markedly increased in patients or in animals experimentally brought under a wide variety of pathological conditions. Significant increases in the glycoprotein content of serum (as hexose or hexosamine) have been shown to be associated with neoplastic diseases, rheumatoid arthritis or infectious diseases. In the present communication, are reported the concentrations of both hexosamine and sero-lactaminic acid in patient

* After this work was finished, the authors found that Dr. Böhm had already reported the isolation of crystalline methoxyl-neuraminic acid from serum (*16*).

sera which are quite in agreement with the results of precedent authors.

The mean normal value, 99 mg. per dl. for hexosamine (as base) observed by us seemed at first sight to be higher than the value generally accepted but is in accord with the results of more recent workers (*20*, *21*), and the mean sero-lactaminic acid content, 78 mg. per dl., agrees well with the value reported by Böhm and his associates as neuraminic acid (*22*).

EXPERIMENTAL, RESULTS AND DISCUSSION

Isolation of Sero-lactaminic Acid—One liter of horse serum was dialyzed against tap water for 3 days, and the remaining sac content (about 1.3 *l.*) was heated for 1 hour with one tenth volume of *N*-sulfuric acid and dialyzed for 4 days against 3 *l.* of distilled water. Sulfuric acid was removed, from the dialysate with Amberlite IR 4B, and the remainder was concentrated *in vacuo* to 200 ml. and passed through Amberlite IRA 400 on which almost the total amount of Bial chromogen adsorbed. After washing with distilled water, the chromogen was eluted with 200 ml. of *N*-sulfuric acid. The eluate was neutralized with baryt, the filtrate was concentrated to a small volume, and chloride present was removed with silver sulfate, the sulfate was precipitated with baryt, and finally the resulting fluid was passed through Dowex 50 column. The concentrated solution was lyophilized, yielding about 200–300 mg. of fluff which appeared sometimes slightly pinkish in color ; it was dissolved in a small volume of absolute methanol, to which was added gradually ether and petroleum ether to faint turbidity. On standing in an ice-box, crystalline needles separated out in a few days. (Fig. 1).

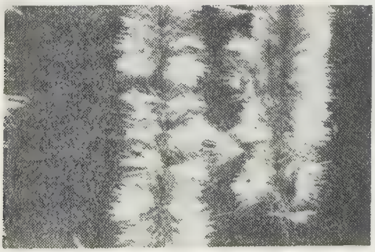

FIG. 1. Photomicrograph of sero-lactaminic acid (about 70×) (taken by Mr. T. Kasai)

*Elemental Analyses**—Although the preparation obtained each time appeared homo-

* Elemental analyses were performed by Mr. B. Kurihara and Miss. E. Kondo.

genous, elemental analyses have not given values consistent enough to establish definitely the composition. The samples were dried at 80° over P_2O_5 under high vacuum to constant weight.

Found : C 42.2–43.5, H 6.5–6.9, N 3.9–4.4, OCH_3 8.5-9.0, CH_3CO 13.0, COOH 13.8.

The most probable composition is $C_{11}H_{19}NO_9$–CH_3OH (mol. wt. 341)
Calculated : C 42.2, H 6.74, N 4.10, OCH_3 9.1, CH_3CO 12.5, COOH 13.2.

Optical Rotation—111.0 mg., 86.5 mg. of the substance dissolved in methanol, water to 3 ml., gave, in an 1 dm.-tube, 3 hrs. after dissolving, a reading of −0.77°, −0.56°, respectively ; hence

$[\alpha]_D^{8°}=-20.8°$ (in methanol), $[\alpha]_D^{8°}=-20°$ (in water).

Decrease of optical activity with time seems to exist, probably due to mutarotation but precise determination could not be achieved. In carring out this procedure, no coloration of the solution appeared as in the case of *N*-acetyl-neuraminic acid of Klenk and Faillard (*14*).

Nature of Sero-lactaminic Acid—The substance obtained from serum is quite similar to Kuhn and Brossmer's lactaminic acid and Klenk and Faillard's *N*-acetyl-neuraminic acid. Each of these substances gave purple color when heated with Bial's orcinol reagent, Dische's diphenylamine reagent or Ehrlich's *p*-dimethylaminobenzaldehyde reagent.

It is very likely that these substances are closely related but not identical with each other. According to the descriptions of German authors, several points of discrepancies are existent.

As shown in Table I, lactaminic acid and sero-lactaminic acid have the equal methoxyl contents and melt under decomposition at the same temperature. *N*-Acetyl-neuraminic acid is, however, almost methoxyl-free. Klenk considered this might be due to dissolving in methanol. The methoxyl content of sero-lactaminic acid is never reduced even after drying over P_2O_5 at 80° under high vacuum, but we considered it would be caused by crystal methanol, because the lyophilyzed fluff before recrystallization procedure is methoxyl-free.

Reducing Power—Lactaminic acid is non-reducing ; *i.e.*, Fehling's solution was not reduced even at boiling temperature and no hypoiodite was consumed according to the method of Macleod and Robison (*23*). *N*-Acetyl-neuraminic acid is reducing ; Trommer's reaction was positive and reducing value was 16.1 per cent (calculated as galactose by Somogyi's method). It consumed more than one mole of iodine with Willstätter-Schudel's aldehyde titration method.

TABLE I

Properties of Sero-lactaminic Acid, Lactaminic Acid and N-Acetyl-Neuraminic Acid

	Sero-lactaminic acid	Lactaminic acid	*N*-Acetyl-neuraminic acid
	per cent	*per cent*	*per cent*
C	42.2–43.5	42.39	42.17
H	6.5–6.9	6.25	6.32
N	3.9–4.4	4.53	4.14
OCH_3	8.5–9.0	8.96	1.7
CH_3CO	13.04	13.71	12.25
COOH	13.8		
Reducing value (as galactose)	21.4	none	16.1
M.p. (decompose)	183–5°	183–5°	
$[\alpha]_D$	−20° (in MeOH,H_2O)	−35° (in MeOH)	−31.7° (in H_2O)
Empirical formula	$C_{11}H_{19}NO_9$–CH_3OH	$C_{11}H_{19}NO_9$	$C_{12}H_{21}NO_{10}$

When heated with Fehling's solution, sero-lactaminic acid produced red precipitates only after 15 minutes just as in the case with the acid hydrolysate of hemataminic acid (*8*) and consumed hypoiodite corresponding to about 10 per cent of glucose under the conditions recommended by Macleod and Robison. Measured by Hagedorn-Jensen's method or ceric sulfate method, the reducing value was ca. 20 per cent (calcd. as galactose). In that occasion, an interesting fact came to light ; with the authentic sugar sample, *e.g.*, glucose or glucosamine-hydrochloride, the reduction of alkaline ferricyanide attained to completion within 5 to 10 minutes but the ferricyanide consumption by sero-lactaminic acid was gradually increased with time as shown in Fig. 2.

10.310 mg. of sero-lactaminic acid consumed 2.521 ml. of NaIO, while under the same conditions 1.490 mg. of glucose consumed 2.791 ml.

Solubility—Klenk's *N*-acetyl-neuraminic acid was crystallized in methanolic solution after addition of a few drops of petroleum ether, but lactaminic acid and sero-lactaminic acid were more easily soluble in methanol and crystallize only after adding a significant amount of ether and petroleum ether ; the similar solubility characteristics was described by Blix in the case of crystallization of sialic acid (*5*).

Color Reactions—Although aqueous solution of acetyl-neuraminic

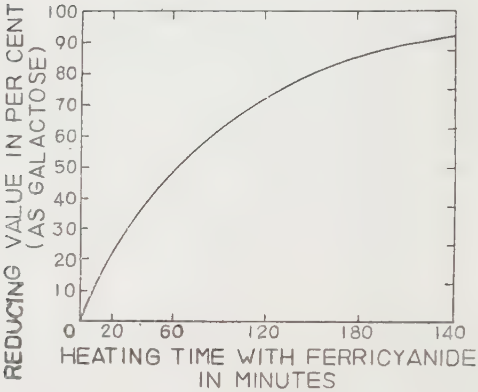

FIG. 2. Reduction of ferricyanide by sero-lactaminic acid by Hagedorn-Jensen's method.

acid became reddish in color with *p*-dimethylaminobenzaldehyde reagent at room temperature, no color appeared in sero-lactaminic acid solution, unless heated. By the orcinol method of Klenk and Langerbeins (*24*), the extinction value of acetyl-neuraminic acid was about 5 per cent higher than the same amount of methoxyl-neuraminic acid, but, measured with sero-lactaminic acid, the value was about 70 per cent of the same amount of hemataminic acid. However, with diphenylamine method, the extinction value of hemataminic acid was much lower than that of the equal amount of sero-lactaminic acid. (Fig. 3).

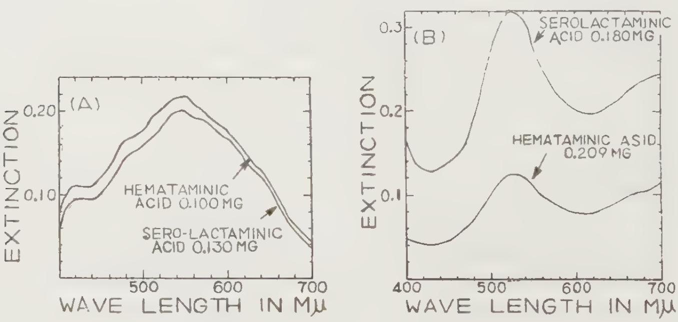

FIG. 3. Absorption spectrum of colored complex with Bial's orcinol (A) and Dische's diphenylamine (B) reagents.

Klenk and Faillard obtained methoxyl-neuraminic acid or hemataminic acid by the methanolysis of acetyl-neuraminic acid and quite recently Böhm and his associates (*16*) isolated it from serum. We could not yet isolate it from sero-lactaminic acid, but a crystalline product was obtained which is yet undetermined. As indicated in absorption spectrum, 2-carboxy-pyrrole was produced from acetyl-neuraminic acid when heated with caustic alkali (*14*); just the similar phenomenon was observed in the case of sero-lactaminic acid.

Column Chromotography of Serum Hexosamine—In general, the procedure of Gardell (*18*) was followed. 0.5 ml. of serum was heated with 0.5 ml. of 4 N HCl for 4 hours in a glass-stoppered vessel. The hydrolysate was evaporated to dryness, the remaining solid mass was dissolved in 0.5 ml. of 0.3 N HCl and chromatographed through a column of Dowex 50 (40 cm. $\times$ 0.6 cm.). The determinations were carried out with human, rabbit and equine sera, as well as Winzler's acid glycoprotein prepared from human plasma. In each case, only one peak of glucosamine was found. (Fig. 4).

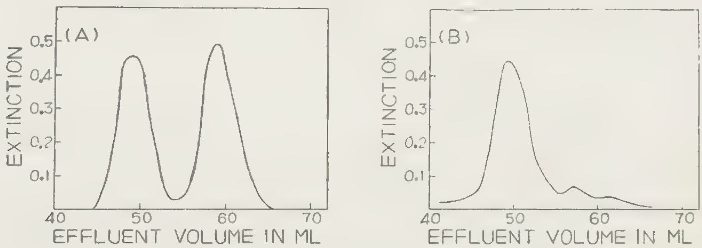

FIG. 4. Separation of hexosamines by ion exchange chromatography.

(A) Mixture of glucosamine (the former peak) and chondrosamine (the latter peak). (B) Serum hydrolysate.

Estimation of Glucosamine and Sero-lactaminic Acid in Normal Animal Sera

Hexosamine was determined by the procedure of Blix (*24*). A sample of 0.05 ml. of serum was heated with 2 N HCl for 14 hours in a boiling water-bath. After cooling, it was neutralized accurately with NaOH and diluted to 2 ml., 2 ml. of acetylacetone reagent (1.5 ml. of pure acetylacetone in 50 ml. of 1.25 N sodium carbonate) was added and the mixture was heated in a boiling water-bath for 45 minutes. After cooling, it was added with 2 ml. of Ehrlich's reagent (1.6 g. of *p*-dimethylaminobenzaldehyde in 30 ml. of 95 per cent ethanol and 30 ml. of conc. HCl) and diluted with 95 per cent ethanol to 25 ml. The determination was made with Coleman electrophotometer at 540 mμ.

Sero-lactaminic acid was determined by the procedure of Klenk and Langerbeins (*24*) for the estimation of neuraminic acid and simultaneously the procedure modified by Werner and Odin (*6*) was carried out. But the results obtained by

either method were essentially the same. For estimation, 0.05 ml. of animal serum was pipetted in a glass-stoppered centrifuge tube, to which 0.95 ml. of water and 2 ml. of Bial's reagent were added and heated in an oil-bath for 5 minutes at 142°. After cooling, 5 ml. of pure iso-amyl alcohol was added and the mixture was vigorously shaken in an ice-bath. After centrifuging, the clear supernatant was removed with a pipette to a cuvette and the optical density was measured against blank with a Coleman electrophotometer using 570 mμ.

The results of analyses are shown in Fig. 5.

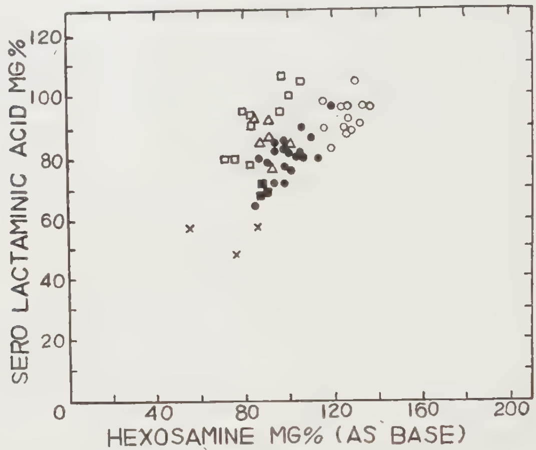

FIG. 5. Sero-lactaminic acid and hexosamine contents in normal animal serum. ● Human, ○ Equine, □ Bovine, △ Guinea pig; ■ Rabbit, × Fowl.

Estimation of Glucosamine and Sero-lactaminic Acid in Pathological States—(Partly performed by Dr. M. Akita of Kawashima Digestive Clinic).

In general, 0.05 ml. of patients sera were used. In cases where the contents were large enough, determinations were carried out with 0.03 ml. The results are summarized in Fig. 6.

The relation of these values to the blood cell sedimentation rate, to electrophoretically determined α-globulin content and to the effects by surgical operation was already reported orally and will be published elsewhere by Dr. M. Akita.

SUMMARY

1. A substance giving purple color with Bial's reagent was isolated from serum mucoprotein in crystalline form and designated as 'sero-lactaminic acid'.

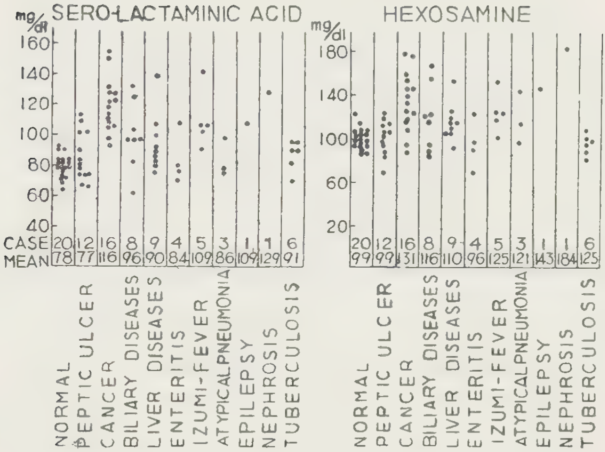

FIG. 6. Serum hexosamine and sero-lactaminic acid in diseases.

2. The nature of sero-lactaminic acid, lactaminic acid and *N*-acetyl-neuraminic acid is discussed comparatively.

3. By the chromatographic technique with cation exchanger, it is revealed only glucosamine and no chondrosamine was present as hexosamine constituent of serum.

4. The contents of sero-lactaminic acid and glucosamine in various animal sera and a variety of patients sera were determined and the increases of both component were noticed in several pathological conditions.

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POSTSCRIPT

1. This work was read at 26th and 27th General Meeting of Japanese Biochemical Society held in Sendai and in Kyoto, in April 1954 and 1955, respectively, and published in Japanese (Medical Science-Seitai no Kagaku, **6**, (5), April, 1955).
2. Dr. L. Odin reported the isolation of sialic acid in human serum (Acta Chem. Scand., **9**, (5), 1955).

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EFFECT OF QUINONE ON THE LIGHT-INDUCED CAPACITY OF CARBON DIOXIDE-FIXATION IN GREEN ALGAE

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(Received for publication, July 25, 1955)

It has been shown in a previous work (*1*) that the light-induced capacity of *Chlorella* cells (in the sence of Calvin and Benson (*2*), for fixing $C^{14}O_2$ in the dark is markedly lowered by the effect of oxygen. By kinetic analysis of the data concerning the formation and disappearance of the capacity in question under various conditions, it was inferred that the level of the capacity is determined by the relative rates of photochemical formation and light-independent inactivation of a certain reducing agent (designated by *R*) which, in normal photosynthesis, fixes and reduces CO_2. Oxygen was proved not to affect the rate of photochemical formation of *R*, but to accelerate the rate of its inactivation, conceivably by oxidizing it irreversibly. It was concluded that both the inhibitory action of oxygen upon photosynthesis (*3*, *4*, *5*, *6*) and the reactivity of oxygen as an oxidant for the Hill reaction (*7*, *8*, *9*) are due to the reaction of oxygen with the substance *R*. If this conclusion be correct, we must expect that a similar phenomenon will also be observed with the Hill reagents other than oxygen.

The present work deals with the effect of quinone, a typical Hill reagent applicable to intact algal cells, upon the fate of *R* under various conditions. It will be shown that, as was expected, quinone affects *R* just in the same manner as oxygen, except that it inhibits irreversibly the reaction between *R* and CO_2 at higher concentrations.

MATERIAL AND METHOD

Experimental material was the "dark cells" of *Chlorella ellipsoidea*, which were grown according to the method of Tamiya *et al.* (*10*). For measuring the light-induced $C^{14}O_2$-fixing capacity of cells, the technique described in the previous paper

(*1*) was employed, using Apparatus I which was designed for following the time course of the effect of pre-treatment of cells. The algal suspension placed in the " lollipop " contained 0.3 ml. (packed volume) of cells in 50 ml. of $M/50$ phosphate buffer of pH 7.0. In this vessel the cells were pre-treated under specified conditions (illuminated* or non-illuminated, and in the presence or absence of quinone) with constant bubbling of N_2.** At various intervals of this treatment, aliquots (3.2 ml.) of cell suspension were promptly transferred into the dark vessels which contained 0.1 ml. each of $NaHC^{14}O_3$-solution (0.03 M). The end concentration of C^{14}-containing $NaHCO_3$ in the vessel was $10^{-3.0}$ M. After allowing the cells to react with $C^{14}O_2$ for 30 minutes, the dark vessel was removed from the apparatus and the cells were immediately killed by introducing 0.5 ml. of concentrated acetic acid-hydrochloric acid-mixture (4:1) into the vessel. To measure the quantity of C^{14} fixed by the cells, 1.8 ml. each of algal suspension, which had been treated with the acid mixture, was transferred to a dish of stainless steel, dried by irradiation with an infra-red lamp, and their radioactivity was determined by using an end-window Geiger-Müller counter. The temperature of experiments was 25°.

RESULTS

Effect of Quinone on the R-Level Established in the Light—In the previous work (*1*) it was shown that when algal cells were illuminated in N_2 atmosphere the light-induced $C^{14}O_2$-fixing power (R) increased at first almost linearly with time and later attained a certain stationary level on prolonged illumination. To investigate whether and in what manner this stationary level is affected by quinone, an experiment reproduced in Fig. 1 was performed. The cells were illuminated, first in the absence of quinone, for 50 minutes—by which time R attained the stationary (maximum) level—and then, under continued illumination, 1.0 ml. of aqueous quinone solution was introduced into the cell suspension.*** At intervals after the addition of quinone, the R-level was determined by measuring the radioactivity fixed in 30 seconds of contact of cells with $C^{14}O_2$ in the dark.**** The final concentration of quinone in the cell suspension was varied in the range between 0 and $10^{-3.7}$ M.

* The effective intensity of light applied was about $2\times 8{,}000$ lux.

** Before the pre-treatment of the main experiment, the cell suspension in the lollipop was aerated in the dark with N_2 for about 30 minutes.

*** Quinone was purified by sublimation, and aqueous solutions were prepared immediately before use, being protected from light before their addition to the cell suspension.

**** For the reason for choosing 30 seconds as the duration of contact of cells with $C^{14}O_2$ in the dark, see our previous paper (*1*).

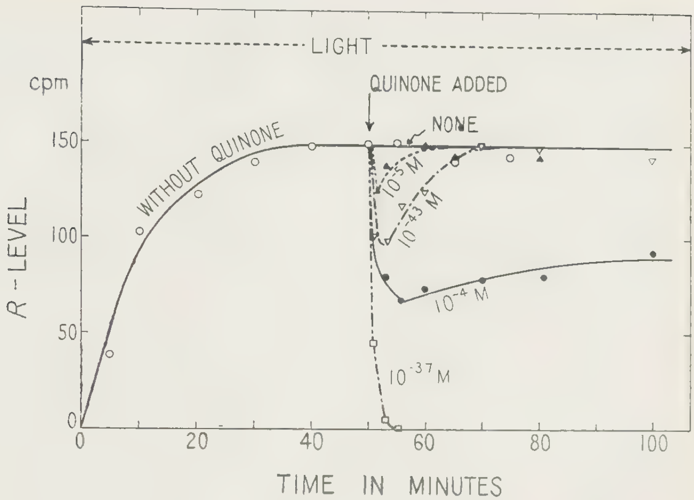

FIG. 1. Effect (in the light) of varying concentrations of quinone on the R-level which had previously attained the stationary (maximum) value in the absence of quinone. Experiments were performed in N_2 atmosphere. Ordinate : count per minute of C^{14} fixed in 30 seconds in the dark. The values of cpm after the 50th minute are those corrected for the dilution of cell suspension caused by the addition of quinone solution. Abscissa : the time which the cells spent in the light (prior to the provision of $C^{14}O_2$), first in the absence of quinone, and later in the presence of various concentrations of quinone as indicated. In this series of experiment the effect of $10^{-4.8}M$ quinone was also investigated. The result obtained fell between those for $10^{-4.3}$ and $10^{-5.0}$ M.

It should be remarked that the final concentration of quinone applied in the ordinary experiments of the Hill reaction is of the order of $10^{-2.3}$—$10^{-3.0}$ M.

As may be seen from the figure, the R-level decreased immediately after the addition of quinone, but in so far as the concentration of quinone was below $10^{-4.3}$ M, it was sooner or later restored to the original value. It is evident that the higher the concentration of quinone applied, the more distinct was the temporary fall of the R-level and the more time was required for its recovery to the original level.

When the concentration of quinone was 10^{-4} M, the temporary fall of the curve was still more pronounced and the subsequent recovery

was only partial. At a still higher concentration of quinone ($10^{-3.7}$ M), the curve fell to the zero level within about 5 minutes, and there was no sign of subsequent recovery on continued illumination lasting as long as 1 hour. To test the reversibility of such an effect of quinone in higher concentrations, an algal sample which had been treated with $10^{-2.7}$ M quinone for 10 minutes was washed 4 times with phosphate buffer, and after an illumination lasting 40 minutes, its capacity for $C^{14}O_2$-fixation in the dark was measured. The result was completly negative, indicating that the $C^{14}O_2$-fixing capacity was irreversibly lost by the treatment of cells with higher concentrations of quinone.

Effect of Quinone on the Decay of R-Level in the Dark—Using $10^{-4.3}$ M quinone, which has been confirmed to cause a reversible suppression of the R-level in the light, its effect on the process of decay of the R-level in the dark was investigated. Algae were illuminated in the absence of quinone for 50 minutes (with bubbling of N_2), by the end of which a stationary R-level was fully established. Then, the light was turned off simultaneously with prompt addition of quinone, and the time course of decay of R-level was followed. As the control the decay of R in the absence of quinone was also traced. From the results presented in Fig. 2 it may be seen that the rate of decay of R in the dark was markedly accelerated by the effect of quinone.

In Fig. 3, the logarithms of the R-level, as it decayed in the dark, are plotted against time. The linearity of the curves indicates that both in the presence and absence of quinone the decay of the R-level proceeded in the manner of a first-order reaction.

DISCUSSION

The experimental data presented above show clearly that quinone reacts with, and is consumed by, the reducing agent R which is formed in algal cells by the effect of illumination. It may be reasonable to consider that the Hill reaction caused by quinone is nothing but a result of the reaction between R and quinone. The fact that, when quinone was applied in lower concentrations in the light, the R-level, after being decreased temporarily, sooner or later restored its original steady value is obviously due to the exhaustion of quinone on continued reaction with R.

It was observed that the $C^{14}O_2$-fixation by algal cells was irreversibly halted when the concentration of quinone applied was higher than $10^{-4.0}$ M. Since the Hill reaction is known to proceed undisturbed in

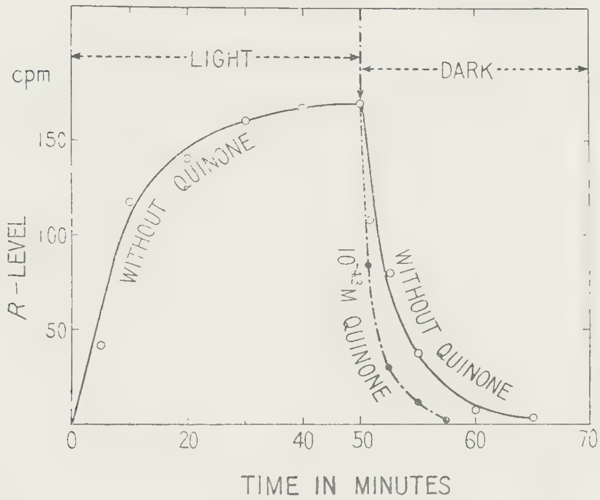

FIG. 2. Effect of quinone ($10^{-4.3}$ *M*) on the decay of *R*-level in the dark. Ordinate : cpm of C^{14} fixed in 30 seconds in the dark. Abscissa : the time which the cells spent in the light and dark, and in the presence and absence of quinone, as indicated, before contact with $C^{14}O_2$ in the dark.

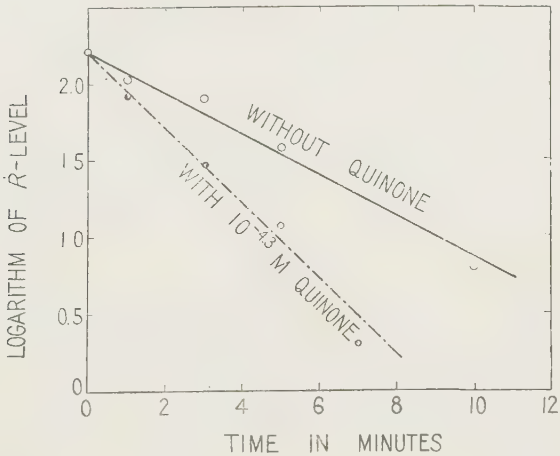

FIG. 3. Semi-logarithmic plot of the process of decay of *R*-level in the dark.

the presence of more than 10^{-3} M quinone,* this fact must be attributed to the destruction of some enzyme system that is functioning in the reaction between R and $C^{14}O_2$. In fact it has been observed by Clendenning *et al.* (*11*) that the photosynthetic CO_2-fixation by algal cells was irreversibly halted by quinone in higher concentrations.

SUMMARY

1. The light-induced $C^{14}O_2$-fixing capacity of algal cells (*Chlorella*), as it was measured by the technique of Calvin *et al.* (*2*, *12*), was found to be markedly decreased when quinone was added to the algal suspension.

2. The suppressing effect of quinone was reversible when its concentration was low ($10^{-4.3}$ M or less), but became irreversible when the quinone concentration was raised (10^{-4} M or more). The reversible suppression of $C^{14}O_2$-fixing capacity caused by quinone in lower concentrations was explained as due to the reaction between the photochemically produced reducing substance and quinone, and the irreversibility of the quinone effect at its higher concentrations was interpreted as being due to the irreversible destruction of the reaction system between the photochemically formed reducing substance and $C^{14}O_2$.

3. It was inferred that the Hill reaction brought about by quinone is nothing but a reaction between quinone and a reducing agent, whose photochemical formation in algal cells can be revealed by the measurement of light-induced $C^{14}O_2$-fixing capacity according to the method of Calvin *et al.*

This work was aided by grants from the Ministry of Education and the Rockefeller Foundation. To these bodies we extend our grateful thanks. Our thanks are also due to Mr. Seikichi Izawa for help with some of the experiments.

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* See, *e.g.*, Clendenning and Ehrmantraut (*11*).

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ON THE VISCOSITY OF HEMOGLOBIN SOLUTION

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(Received for publication, July 26, 1955)

In his recent study on the dielectric properties of hemoglobin-oxyhemoglobin-system, Takashima (*1*) has found that the value of dielectric increment (∂) of hemoglobin solution (in distilled water) shows a characteristic change with the progress of oxygenation. The plot of ∂ against oxygen partial pressure gave a distinctly double-peaked curve, indicating that there are 4 stages of oxygenation which are accompanied alternately by the increase and decrease of dipole moment. Several explanations might be suggested to account for this highly interesting observation ; namely :

(1) the shape of hemoglobin molecule was changed with the progress of oxygenation,

(2) oxygenation was accompanied by alternate association and dissociation of hemoglobin molecules,

(3) with the progress of oxygenation, some changes such as the shift of pH in the solution occurred, as a result of which the dielectric properties of hemoglobin was modified,

(4) the distribution of electric charge in the hemoglobin molecule was profoundly modified by the effect of oxygen attached to hemes.

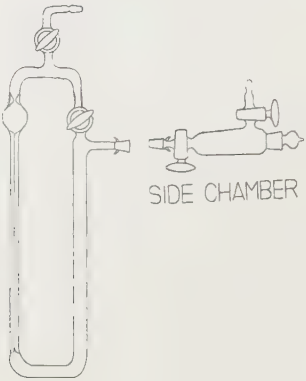
In cases (1) and (2), the change is expected to be accompanied by a change of viscosity of hemoglobin solution, and in case (3) a complicated variation of pH must be demonstrated directly by experiment. The present study was carried out with a view to elucidating these points.

EXPERIMENTAL

The material used was recrystallized horse oxyhemoglobin which was prepared according to Ferry and Green (*2*). In viscometric experiments it was dissolved in 0.2 M phosphate buffer of pH 6.8, and after centrifuging off undissolved materials and filtering through a glass filter, the solution was made up with distilled water to contain 0.1 M phosphate. Use was made of a capillary viscometer of Ostwald type, which is

illustrated in Fig. 1. The bulb had a volume of about 4.0 ml., and the capillary was of the dimension that the flow time of water was 1098 seconds at 25. 0°. In the side chamber shown in the figure, 10 ml. of the hemoglobin solution in phosphate buffer was placed, and it was deoxygenated by repeated evacuation and equilibration with N_2,

Fig. 1



taking caution to avoid the formation of bubbles. When the completion of deoxygenation was ascertained by disappearance of oxyhemoglobin band (541 and 577 mμ) and appearance of reduced hemoglobin band (555 mμ), the solution was equilibrated with N_2-O_2-mixture of known O_2 concentration. Then, the viscometer was filled with the same gas mixture, and after the whole apparatus was dipped in the water of thermostat, the solution was introduced into the iscometer and its flow rate was determined. The temperature of experiment was $25.0 \pm 0.05°$.

The concentration of protein was determined by the micro-Kjeldahl method. In Fig. 2 are plotted the specific viscosities of reduced and O_2-saturated hemoglobins, and methemoglobin as they changed linearly with the protein concentration. The " reduced viscosity " (or " viscosity number ") was calculated by dividing the specific viscosity by the concentration in grams of protein/100 ml. solution. The results presented in Fig. 3 shows clearly that the viscosity of the hemoglobin solution was not modified at all during the process of oxygenation.

The change of pH of hemoglobin solution which occurred concomitantly with the process of oxygenation, was investigated by the following procedure. Oxyhemoglobin was dissolved in distilled water, and placed in contact with air in a stoppered glass vessel which was provided with glass and calomel electrodes for pH-measurement. Keeping the equilibration between the solution and gas mixture, the air was gradually replaced with N_2 until all hemoglobin became deoxygenated. During this process the pH of the solution was found to shift linearly by $+0.21$ unit (the Bohr effect) showing no sign of double-peaked fluctuation as it was observed by Takashima in the change of dielectric moment.

FIG. 2

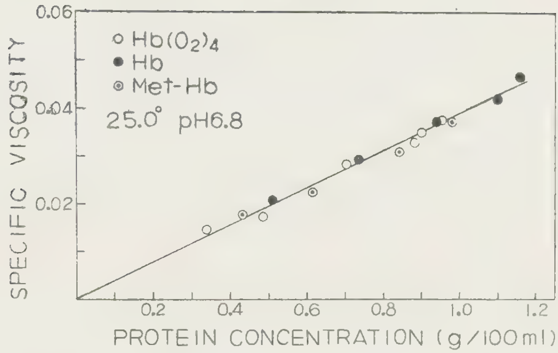
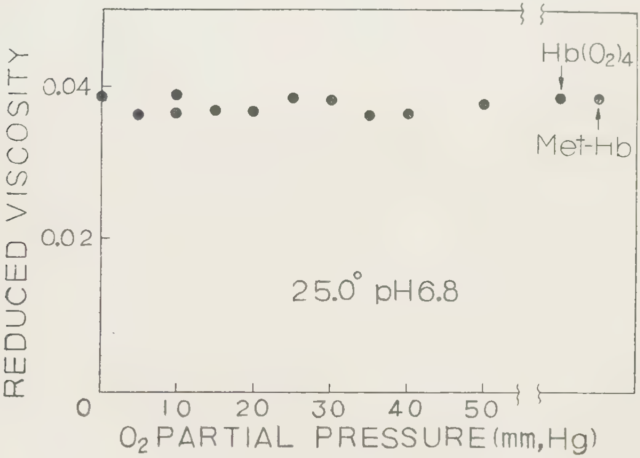


FIG 3



SUMMARY AND DISCUSSION

Our observation that the viscosity of hemoglobin solution remained the same under different oxygen partial pressures allow us to conclude that—in so far as the hydration and the partial specific volume of hemo globin molecule are assumed to remain unchanged in the process of oxygenation—the axial ratio of hemoglobin molecule is not modified by the effect of oxygenation. It was also confirmed that in the course of oxygenation the pH value of hemoglobin solution shifted steadily

in a manner as it was expected from the Bohr effect. The fact discovered by Takashima (*1*) that the dielectric moment of hemoglobin shows a characteristic doublepeaked fluctuation in the course of oxygenation must be attributed to the modification of charge distribution on the globin part, which was brought about by the effect of structural change in oxygenated hemes.

The auther acknowledges his indebtedness to Prof. H. Tamiya for his encouragement throughout the course of this work.

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PROPERTIES OF PURIFIED YEAST CYTOCHROME c

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Cytochrome c of baker's yeast was the first one that was isolated and its properties were studied to some extent by Keilin (*1*). But after Theorell (*2*), and Keilin and Hartree (*3*) succeeded in preparing the pigment in higher state of purity from mammalin heart muscle, most of studies on cytochrome c were done with the preparations obtained from heart muscle, while those from other sources were seldom employed. Thus, there remains many questions to be solved concerning the properties of yeast cytochrome c, such as the oxidation-reduction potential, which is claimed by some to be very close to that of heart cytochrome c while by others to be distinctly lower.

Recently Neilands (*4*) pointed out the lack of information about species specificity of respiratory pigments and compared the properties of pure cytochrome c isolated from rust fungus (*Ustilago sphaerogena*) with those of heart muscle cytochrome c. The spectrum of both preparations was in good agreement reflecting the identity of the prosthetic group, while properties of protein moiety such as isoelectric point and molecular weight were different.

Accordingly, it was undertaken to isolate the yeast cytochrome in pure state and to investigate its absorption spectra, oxidation-reduction potential, chromatographic behavior and paper electrophoretic patterns. The results of these studies are reported in this paper.

EXPERIMENTAL

Isolation of Cytochrome c from Baker's Yeast—Commercial press yeast supplied from Oriental Yeast Co. was used. The method of extraction was essentially the same as that described by Keilin (*1*). The extract was dialysed against running tap water overnight, and purified by the use of cation exchange resin as described by Margoliash (*5*), where a column of ammonium salt of Amberlite XE 64 of dimension 10 $\times$ 1.5 cm. was used. The column on which the protein was adsorbed, was washed with water and followed by ammonium acetate solution. The pigment was eluted with

0.25 N ammonia and dried from frozen state, wereby ammonia was drived off. Purity of this preparation was estimated by extinction of ferrocytochrome c at 550 mμ, assumming that the molar extinction coefficient is the same with that of heart cytochrome c ($\varepsilon \times 10^{-5}$ 0.28). The calculated iron content of this preparation was 0.36 to 0.37 per cent.

Preparation of Cytochrome c from Heart Muscle—The procedure was essentially the same as described by Keilin and Hartree (*6*) except that trichloracetic acid was replaced by 0.15 N perchloric acid. Precipitated cytochrome c was dissolved in a volume of water to make the salt concentration 0.01 to 0.03 M and purified by ion exchange resin as described above.

Spectrophotometric Measurements—Absorption spectra of the preparations were determined by Hitachi Model EPU-1 quartz spectrophotometer. Reduction of the pigment was accomplished with hydrogen and paladium black, and oxidation by aeration at pH 1.

Measurements of Oxidation-Reduction Potential—Ferroferricyanide oxidation-reduction buffers were employed for the estimation of oxidation-reduction potentials as described by Davenport and Hill (*7*). The buffer solutions were prepared just before use.

*Paper-Electrophoresis**—Toyo filter paper, No. 3, approximately 5×30 cm. in size was used and a potential of 200–300 volts was applied to give a current of 1.2 mA per cm. width.

Ion Exchange Chromatography—The column used was the same as that for preparation of the pigment. The pigment adsorbed at the top of the column was developed with 0.25 N ammonium acetate solution, 0.5 M ammonium acetate-ammonium hydroxide buffer of pH 9.6 and 0.25 N ammonia. The successive portions of the effluent were analyzed for cytochrome c concentration spectro-photometrically after reduction with sodium dithionite.

Measurement of Catalytic Activities—The catalytic activities of the preparations were examined in the rat liver succinic dehydrogenase system of Potter (*8*). The concentration of cytochrome was estimated by extinction of ferrocytochrome c at 550 mμ.

RESULTS

Absorption Spectrum—Figs. 1 and 2 show the complete spectra of yeast cytochrome c in reduced and in oxidized form respectively, along with the spectrum curves reconstructed from the authentic data for heart muscle cytochrome c by Theorell (*2*). In the figures the height of the curves for ferrocytochrome c at 550 mμ has been equalized. The spectra in visible region were almost identical with those of heart cytochrome c, reflecting the identity of the prosthetic group of the pigment.

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* The author is indebted to Drs. K. Shimao and H. Sato of the Department of Biochemistry, for their kind advices.

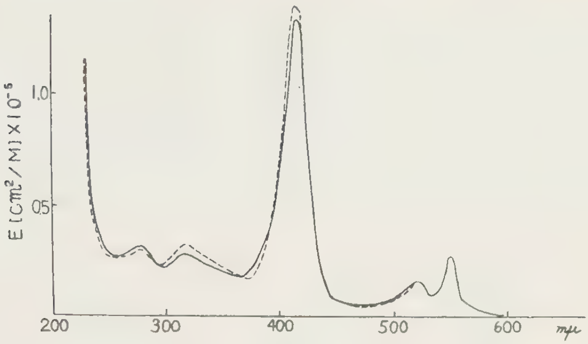

FIG. 1. Absorption spectra of reduced cytochromes.
Solid line, yeast cytochrome c, reduced with hydrogen and palladium black ; broken line, ferrocytochrome c, from Theorell (*2*).

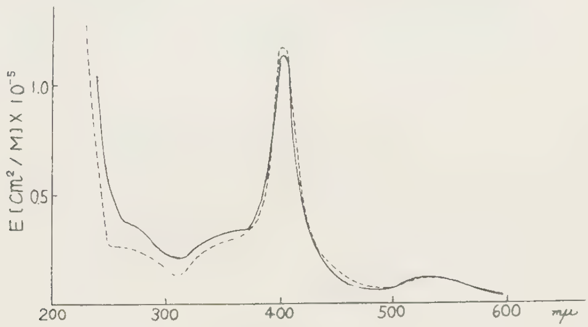

FIG. 2. Absorption spectra of oxidized cytochromes.
Solid line, yeast cytochrome c oxidized by aeration at pH 1 ; broken line, ferricytochrome c, from Theorell (*2*).

Even the ultraviolet absorption curves of both preparations demonstrated the same general contour which indicated the similarity in structure of the protein moieties.

Morever, yeast cytochrome c showed the same behavior against oxidation and reduction at low and high pH as the heart preparation Neither autoxidation nor binding with cyanide or carbon monoxide occurred at physiological range of pH.

Oxidation-Reduction Potential—The ratio of oxidized to reduced cytochrome c in ferro-ferricyanide oxidation-reduction buffer is shown in Fig. 3. As is seen in the figure, in both preparations a one-electron

change was involved in the oxidation, and the oxidation-reduction potentials were almost identical.

Chromatography—Yeast cytochrome c was readily adsorbed on ammonium form of Amberlite IRC 50, and eluted by raising the salt concentration, just as the heart cytochrome c. Fig. 4 presents the chromatograms of the cytochrome preparations from yeast and from heart muscle eluted at pH 7.0 and pH 9.6.

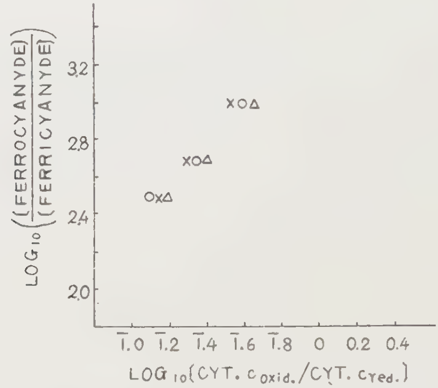

FIG. 3. The ratio of oxidized and reduced cytochrome c in ferro-ferricyanide oxidation-reduction buffers (about 10°).

× heart cytochrome c (pH 7.0), ○ yeast cytochrome c (pH 7.0), △ yeast cytochrome c (pH 6.0).

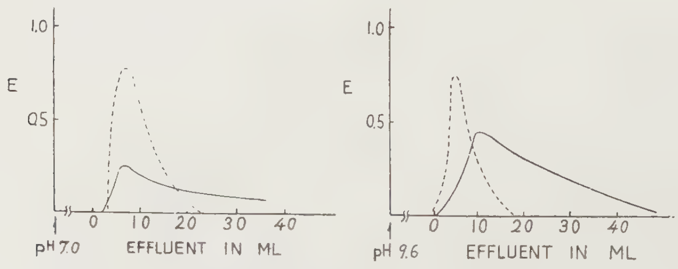

FIG. 4. Elution chromatograms of cytochrome c.

Column of Amberlite XE 64 ; ammonium salt, 1.5×12 cm. Rate of flow 0.8–1.0 ml./min. Total of 0.2 M of cytochrome c adsorbed on the column. Elution with 0.25 N ammonium acetate solution (pH 7.0) and 0.5 M ammonium acetate-ammonium hydroxide buffer pH 9.6. Solid line, yeast cytochrome ; broken line, heart cytochrome.

When mixture of both preparations was chromatographed using buffer solution of pH 9.6 and 0.25 N ammonia as eluting solvent, distinct sign of separation of the two components could not be demonstrated (Fig. 5). The only difference observed was that yeast cytochrome c emerged in a fairly wider band than that of heart muscle.

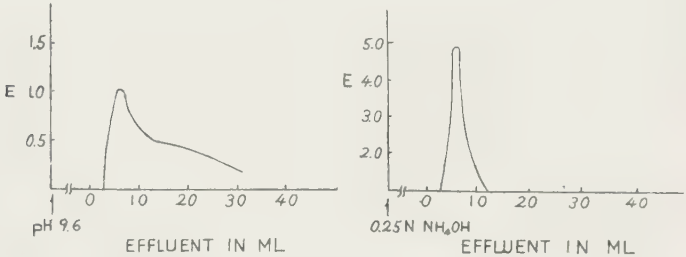

Fig. 5. Elution chromatograms of mixtures of cytochromes.
Elution with ammonium acetate buffer pH 9.6 (the same with in Fig. 4) and 0.25 N ammonia. Column, rate of flow and abbreviations as in Fig. 4.

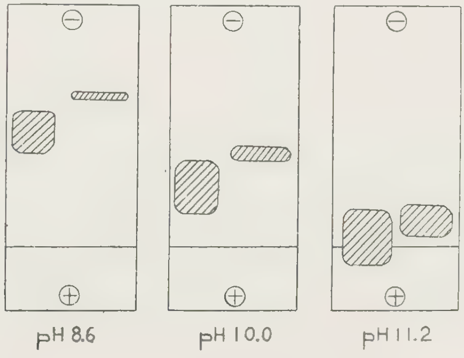

Fig. 6. Paper electrophoresis of cytochromes.
Toyo filter paper No. 3. (5×30 cm.). 3 hours with a potential of 200–300 volt, current of 1.4 mA/cm. width. Buffers : veronal (pH 8.6, $\mu=0.1$), 0.05 M sodium carbonate-sodium bicarbonate (pH 10.0) and 0.05 M sodium carbonate (pH 11.2). Left, yeast cytochrome ; right, heart cytochrome.

Paper-Electrophoresis—The tendency of movement at various pH was the same in both preparations (Fig. 6). From the results of paper-

electrophoresis near the isoelectric point of heart cytochrome c (pH 10.6), the isoelectric point of yeast cytochrome c was estimated to be very close to that of the heart cytochrome c. The diffuse band resulted from electrophoresis of the yeast preparation, is attributable to microheterogeneity in electrophoretic mobility of the preparation and would be related to the wide band by ion exchange chromatography.

Catalytic Activities—Table I shows the catalytic activities of both yeast and heart preparations.

TABLE I

O_2 Uptake in the Rat Liver Succinic Dehydrogenase System (8)

Concentrations of cytochrome c added *M*	O_2 uptake per hour ($\mu l.$)	
	Yeast cytochrome	Heart cytochrome
not added	45	45
1×10^{-5}	95.5	97
3×10^{-5}	165	179
5×10^{-5}	166	181

DISCUSSION

Differences between c-cytochromes of different origin are believed to be reflected in the ways they are prepared (*9*). In the case of the yeast cytochrome, the same would be true. Trichloracetic acid extraction which is excellent for heart cytochrome c completely failed for this pigment from yeast. Dilute sodium hydroxide and secondary phosphate, which were preferred to acid for extraction from rust fungus and wheat germ (*10*) respectively gave also low yields. This evidence reflects differences in the chemical or physical bondings of these cytochromes to cell structure.

From the difference of protein parts of cytochromes, difference of the oxidation-reduction potential is plausible. Coolidge was the first who determined the oxidation-reduction potential of yeast cytochrome c in crude state and reported the value of 0.27 volt at pH 7. But according to the results of other workers the E_0' values for yeast cytochrome c are distinctly lower than those for mammalian cytochrome c ($E_0'=0.12$ volt according to Green (*11*), de Toeuf (*12*) and Baumberger (*13*)). To these measurements several objections arose (*9*) and from author's

experiments, oxidation-reduction potential of purified yeast cytochrome c was determined to be almost identical with that of the heart preparation.

From the present findings the similarity of yeast and heart cytochromes may be stressed. *Ustilago* cytochrome c has quite different isoelectric point and "cytochrome c" of photosynthtic bacteria (*Rhodospirillum rubrum*) differs in its electrophoretic, adsorption and enzymatic properties (*14*). The close resembrance of ultraviolet absorption curves and isoelectric points between yeast and heart cytochromes indicates the similarity of protein moieties. Wide and diffuse band which was formed during electrophoresis and chromatography of yeast cytochrome remind the author of the spreading of the electrophoretic boundaries of cytochrome f observed by Davenport and Hill (*7*).

SUMMARY

1. Cytochrome c of baker's yeast was purified by ion exchange resin chromatography.

2. Absorption spectrum, oxidation-reduction potential, isoelectric point and enzymatic activities of both yeast and heart cytochrome c were closely similar.

3. Similar chromatograms and paper-elctrophoreses of both cytochrome c were obtained, but yeast cytochrome showed wider bands by chrmatography and diffuse boundaries by electrophoresis.

The author expresses his thanks to Prof. H. Yoshikawa and D. Y. Yoneyama for constant guidances and Dr. M. Ishimoto, Department of Chemistry, Faculty of Science, and Dr. J. Koyama, Institute for Infectious Diseases, for their constant advices and encouragements in the course of the work. This work was aided by a grant from the scientific research fund of the Ministry of Education.

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SEPARATING DETERMINATION OF RIBOFLAVIN NUCLEOTIDES BY PAPER ELECTROPHORESIS

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(Received for publication, July 30, 1955)

The senior author (*1*) reported the separating determination of riboflavin nucleotides in animal tissues using both paper partition chromatography and the lumiflavin-fluorescence method. Since the quantity of flavin compound was relatively small in animal tissues, the absolute quantity of each flavin nucleotide was calculated from the total quantity and their ratio to each other determined from a concentrated solution.

However, a relatively large amount of flavins will be separated from each other more rapidly on a filter paper by the application of an electric current or a potential gradient (*2*, *3*), and, therefore, paper electrophoresis can be applied to the separating determination of flavins. From this point of view, quantitative examinations were made on paper elecrophoresis of flavins and the procedure is described in this paper.

Materials

Flavins—Free riboflavin (FR) and flavin mononucleotide (FMN) were furnished by the Hoffmann-La Roche Co. FMN was purified by electrophoresis on a thick filter paper (Toyo Roshi No. 26 H), because a small amount of FR and other derivatives were present in it.

Flavin-adenine dinucleotide (FAD) was extracted from Eremothecium ashbyii and purified by circular paper chromatography (*4*) and "Florisil" (*5*) followed by paper electrophoresis as in the case of FMN.

Apparatus Used for Paper Electrophoresis—The design of apparatus and the technical details followed those described by Kobayashi (*6*). The filter paper moistened with a buffer solution is held horizontally on a plastic holder, and bridged between two plastic baths having two compartments. Both ends of the paper strip is dipped into the buffer solution in the inside compertment as shown in Fig. 1. The outside compartments of the baths are filled with 5 per cent KCl solution and the platinum electrodes are placed in them. The solutions in both compartments are made up to the same level and these are electrically connected by agar bridge. The electrodes are connected to the electric current-regulator.

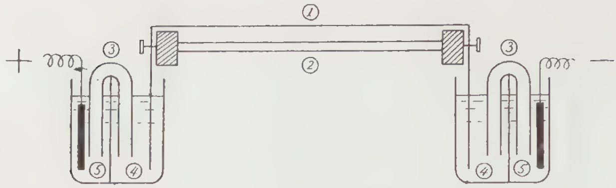

Fig. 1. Apparatus for paper electrophoresis
① Filter paper strip, ② Paper strip holder, ③ Agar bridge, ④ Buffer solution, ⑤ 5% KCl solution.

Experimental

Paper Electrophoresis of Riboflavin Nucleotides—Prior to electrophoresis, the filter paper (Toyo Roshi No. 50, 12×23 cm.) was moistened with $M/20$ phosphate buffer solution of pH 8.0 and the current of 2.44 mA per cm of paper width was applied during 10 minutes (at 12 cm. width, a current of 30 mA was required per strip). Then flavin solution was applied to the filter paper at the line 8 cm. from the edge of the anode side. For the quantitative estimation, 0.1–0.2 ml. of flavin solution should be applied as a long band on the paper.

The electrophoresis was carried out in a dark chamber. When a current of 2.44 mA per cm. of paper width had been applied for about 15 minutes, FR, FMN and FAD moved toward the cathode with different mobilities and the difference of mobilities of these compounds became distinct after 2 hours of electrophoresis. The migrated zone of flavins, as shown in Fig. 2, was observed under the ultraviolet light passed through a UV filter. The mobilities of these flavins at different periods are shown in Table I.

Table I

Mobility of Flavins on A Filter Paper in Electrophoresis

($M/20$ Phosphate buffer, pH 8.0; 2.44 mA/cm.)

Time	Mobility of flavins (toward the cathode) (*mm.*)		
hrs.	FR	FMN	FAD
0.25	2	16	12
0.50	4	25	20
1.00	7	35	28

The zone of flavins after electrophoresis was separated more clearly in weakly alkaline than in acid medium. The decomposition of FAD or FMN to FR during

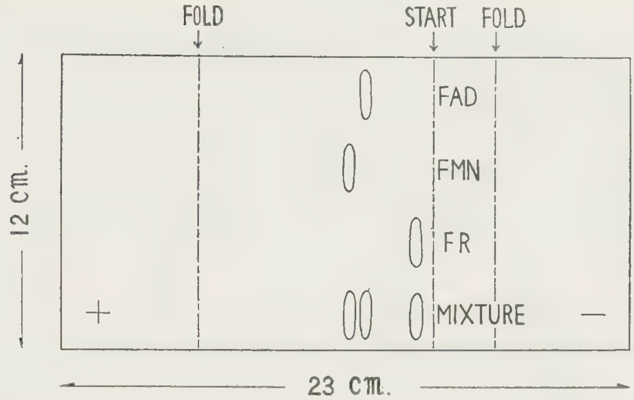

FIG. 2. Separation of flavins by paper electrophoresis
$M/20$ phosphate buffer pH 8.0, current 2.44 mA/cm., after 2 hours.

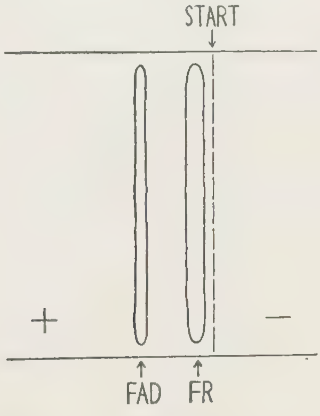

FIG. 3. Separation of flavins in the aqueous extraction of *Eremothecium ashbyii* by paper electrophoresis

0.2 ml. of aqueous extract was applied on the paper for quantitative determination.

the paper electrophoresis was not recognized.

Procedure for Quantitative Estimation—After the electrophoresis, the portion of the filter paper containing flavins was cut out with blank spaces around the zone. The paper strip was cut into small pieces, placed in a glass-stoppered, graduated tube of 10 ml. capacity, 9 ml. of water was added, and warmed at 80° for 15 minutes, with occasional agitation. After being allowed to cool, the volume of the eluate was brought

TABLE II

Recovery of Flavins by Paper Electrophoretic Method in A Sample Containing One Component of Flavin

(M/20 phosphate buffer, pH 8.0; 2.44 mA/cm. Flavin was determined by spectrophotometry and by the lumiflavin fluorescence method.)

Flavins	Expt. No.	Duration of electrophoresis (*hrs.*)	Amount applied (γ)	Amount recovered (γ)	Recovery rate (%)
FR	1	1	8.5	8.7	102.0
	2	1	17.0	17.9	105.0
	3	2	25.5	25.7	100.7
FMN	1	1	40.0	39.9	99.9
	2	1	69.7	70.0	100.2
	3	2	161.8	162.7	100.6
FAD	1	1	47.9	50.0	104.0
	2	1	95.8	95.2	99.2
	3	2	236.0	239.0	101.2

TABLE III

Recovery of Flavins by Paper Electrophoretic Method in A Sample of Flavin Mixture

(M/20 phosphate buffer, pH 8.0; 2.44 mA/cm. Flavin was determined by spectrophotometry)

Flavins	Amount applied (γ)	Amount recovered (γ)	Recovery rate (%)
FR	20.0	20.2	102.0
FMN	153.0	151.0	98.6
FAD	120.0	125.0	104.2
Total flavin (as FR)	203.9	204.8	100.4

to 10 ml. with water and filtered.

If the flavin concentration of the eluate was over 1 γ/ml., calculated as FR, light absorption at 450 mμ was measured by the Beckman spectrophotometer and flavin concentration was calculated from the molar extinction coefficient of each flavins at 450 mμ. To measure the light absorption, the solution for the blank test was prepared by the above described procedure from a strip of paper which was not spotted with flavin or other dyes.

When the flavin concentration of the eluate was low, flavin was determined by

the lumiflavin fluorescence method using a microphotofluorometer (*7*). In this case, the riboflavin fluorescence method was not suitable because the true fluorescent energy cannot be measured in the presence of a fluorescent substance extracted from the paper or in the presence of a fluorescence quencher, for instance, inorganic ion and so on.

Recovery Test—The recovery of the flavin compounds in the method described above is shown in Table II, in which the sample of one component of flavin was used, and in Table III, where a mixed sample was used. The results showed that the rccoveries are within 100±5 per cent.

DISCUSSION

The separating determination of riboflavin nucleotides by electrophoresis on a filter paper was very rapid compared with the method using paper chromatography. Moreover, as a considerably large amount to flavins can be separated by this method, this is considered to be suitable for the analysis of partially purified flavin nucleotides or other samples containing a large amount of flavins.

While this work was in progress, Siliprandi *et al.* (*3*) reported the separation of flavin compounds by paper electrophoresis using acetate buffer of pH 5.1, and Peel (*8*) described its application to the determination of flavins in microörganisms as a short report.

For the separating determination of flavins in animal or vegetable tissues, the following procedure similar with the previous report (*1*) should be adopted, because the tissues of organism contain flavins generally in minute concentrations. First, the total flavin content was measured by the lumiflavin-fluorescence method, secondly, the ratio of each flavin compound was measured from the concentrated flavin solution by the above mentioned method, then the content of each flavin was calculated from these two estimations.

SUMMARY

Flavin compounds could be separated by virtue of different mobilities in the electrophoresis on the buffer-moistened filter paper. Each flavin was eluted with water, and their concentration was determined by measuring the light absorpion of the cluate at 450 mμ or by the lumiflavin fluorescence method. The recovery by this method was satisfactory, being 100± 5 per cent.

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ASPARAGINE METABOLISM IN THE GERMINATION STAGE OF A BEAN, VIGNA SESQUIPEDALIS*

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(Received for publication, July 30, 1955)

The systematic investigations on the growth process of a legume, *Vigna sesquipedalis*, in the germination stage are now proceeding in this laboratory (*1–7 etc.*). The present paper aims at interpreting the seedling growth in connection with the behavior of water soluble nitrogen compounds. Because of a marked accumulation of asparagine in the bean seed embryo, particular attention is paid to the amide.

The part played by asparagine in the nitrogen metabolism in higher plants has not yet been clarified exactly and is, in general, assumed to be merely of secondary significance, *i.e.*, as storage or transport form of nitrogen. Recent reports of Meister *et al.* (*8*, *9*) on hepatic preparations, however, suggest a direct contribution of asparagine to the amino acid and protein syntheses. According to him the α-amino group of the amide can be transferred to various α-keto acids with the participation of asparagine transaminase. It will be reported below that a similar mechanism of α-keto acid-dependent deamidation of asparagine may be operating in the seed embryo, and various other findings which will serve to stress the importance of asparagine in the growth metabolism in question will also be described.

MATERIALS AND METHODS

Bean Seeds—Seeds of *Vigna sesquipedalis* harvested in 1953 in the farm of this laboratory were used. The seeds were germinated in the dark at 30°. Detailed description of the methods of germination and separation of organs from seedling plants of various ages has been made elsewhere (*1*). As the 0 day-old material, seeds soaked in water for 5 hours at 30° were employed.

Water Extract—Tissues were homogenized by means of a glass homogenizer with

* The compendium of this work was announced at the Annual Meeting of the Botanical Society of Japan held in Kyoto on Oct. 26, 1954.

* As for the definition of the germination stage, see Oota *et al.* (*1*, *2*).

an appropriate amount of phosphate-borate buffer ($M/10$, pH 6.5), and centrifuged for 15 minutes at $1,100 \times g$. The sediment was extracted with the buffer twice more. The supernatants were combined, and used for the determination of various nitrogen compounds.

Total Soluble Nitrogen—Total nitrogen was estimated by the method of Levy and Palmer modified by Yagi (*10*).

Ammonia, Glutamine and Asparagine—Ammonia-N, glutamine amide-N, and asparagine amide-N were assayed by the method of Vickery *et al.* (*11, 12*).

Total α-Amino Nitrogen—This was estimated by the method of Pope and Stevens (*13*). Amide group of asparagine did not interfere with this amino estimation.

Identification of Amino Acids and Amides—One dimensional paper chromatography was applied to the qualitative examination of both amino acids and amides, using phenol+water (85:15) as the developing solvent.

Soluble Protein—The water extract was treated with 10 per cent trichloroacetic acid. The sediment was washed with 5 per cent. trichloroacetic acid. Total nitrogen content of the residue was estimated by the above-cited modification of Levy and Palmer's method and was regarded as protein-N.

Adenosine Triphosphate (ATP)—ATP was prepared by the method of Szent-Györgyi (*14*) using fresh skeletal muscle of a dog killed by the $MgSO_4$ injection (*15*).

Reagents—α-Ketoglutaric acid was synthesized by the method described in Umbreit *et al.* (*16*). Other reagents used were purchased in a market*. Free organic acids were neutralized to pH 6.5 with NaOH prior to use.

RESULTS AND DISCUSSION

Concentration Gradient of Asparagine in the Embryo

Changes in quantity of asparagine amide-N, glutamine amide-N, ammonia-N, amino-N, and total soluble-N in the water extract of each embryonic organ in the germination stage were investigated. Results of a typical run are illustrated in Figs. 1–5.

In these figures the features of respective organs as to the nitrogen metabolism are clearly shown. The cotyledons of O day-old seedlings already contain a large quantity of asparagine, which amonuts to about 35 per cent of total soluble nitrogen. As the seeds begin to germinate, the asparagine content in the cotyledons drops markedly. Amino-N content also decreases gradually after passing a slight peak on the 2nd day of cultivation (Fig. 1). The hypocotyls continue to accumulate asparagine and amino compounds up to the 4 or 5th day (Fig.

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* Pyridoxamine (Merck) was provided from Prof. Y. Hirata of the Chemical Institute of this Faculty. The author wishes to express thanks to him.

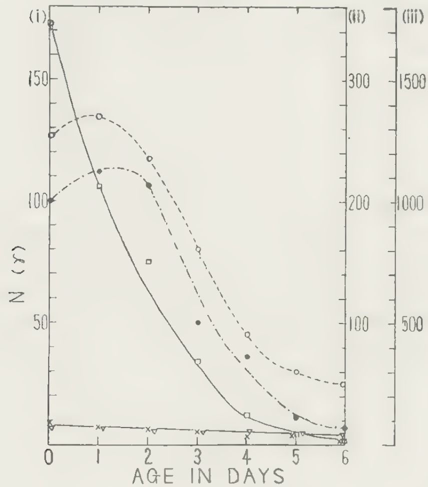

FIG. 1. Changes in quantity of water soluble nitrogen compounds in cotyledons of *Vigna sesquipedalis* in the germination stage.
—□—: Asparagine amide-N plotted on scales (i)
—×—: Glutamine amide-N plotted on scales (i)
—▽—: Ammonia-N plotted on scales (i)
---○---: Amino-N plotted on scales (ii)
-·-●-·-: Total soluble-N plotted on scales (iii)
Values per a pair of cotyledons are plotted.

2), while in the radicles and the plumules rather indistinct changes in asparagine are observed at least in the early germination stage (Figs. 4 and 5).

It is noticed that the asparagine content of the hypocotyl declines markedly in the later germination stage, when the epicotyl growth proceeds and the asparagine content of the epicotyl increases evidently (Figs 2, 3).

The hypocotyls of the 3 and 6 day-old plants were divided into three parts, *i.e.* lower, middle, and upper parts after Uemura (cited in (*6*)) who had shown that the elongation growth of these three parts is completed by the 3rd, 4th, and 5th day, respectively and thus the growth pattern apparently translocates upwards in the stem axis. Recently, Izawa (unpublished), measuring the longitudinal distribution of total (water soluble and insoluble) protein-N in hypocotyl, has suggested that this acropetal translocation of growth pattern

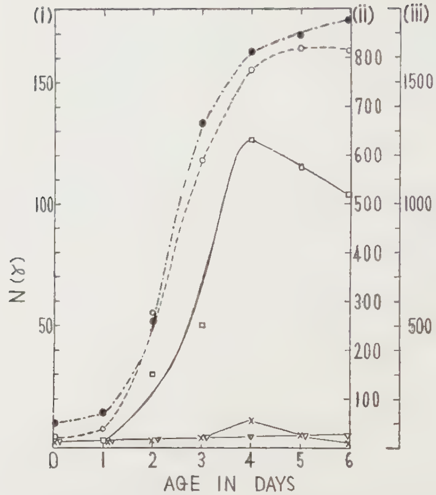

FIG. 2. Changes in quantity of water soluble nitrogen compounds in hypocotyls of *Vigna sesquipedalis* in the germination stage.

See text of Fig. 1 for symbols and scales used. Values per hypocotyl are plotted.

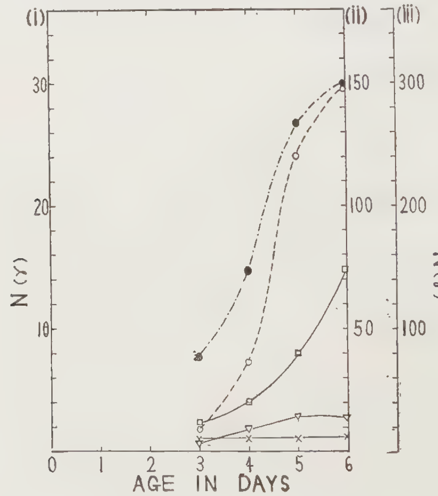

FIG. 3. Changes in quantity of water soluble nitrogen compounds in epicotyls of *Vigna sesquipedalis* in the germination stage.

See text of Fig. 1 for symbols and scales used. Values per epicotyl are plotted.

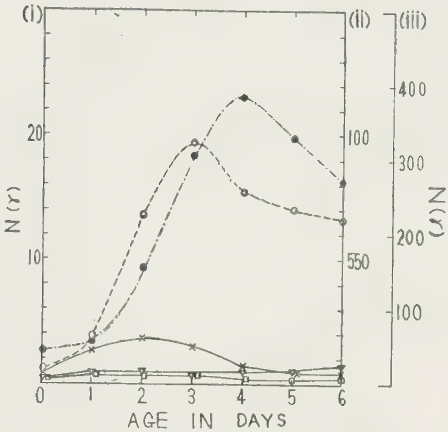

Fig. 4. Changes in quantity of water soluble nitrogen compounds in radicles of *Vigna sesquipedalis* in the germination stage.

See text of Fig. 1 for symbols and scales used. Values per radicle are plotted.

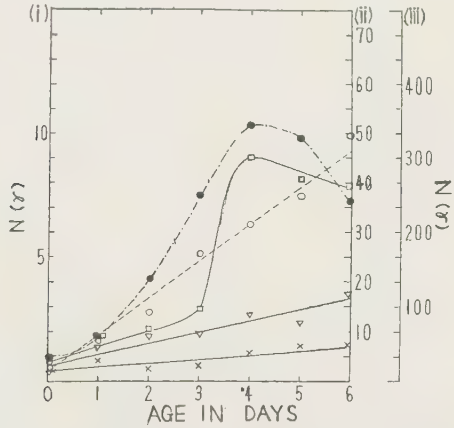

Fig. 5. Changes in quantity of water soluble nitrogen compounds in plumules of *Vigna sequipedalis* in the germination stage.

See text of Fig. 1 for symbols and scales used. Values per a pair of plumules are plotted.

is accompanied by that of protein formation ability. The water extract was prepared from each part and the changes in contents of nitrogen

compounds were investigated. As shown in Table I, the lower part of the 3 day-old whose elongation growth has just ceased to proceed is abundunt in asparagine. The upper part of the same age, which is performing vigorous elongation, however, contains no asparagine, while is rich in ammonia, amino acids, and glutamine. Thereafter, with the proceeding of age (see the column for the 6 day-old), the asparagine peak translocates to the upper part that is then losing its growth activity.

TABLE I

Changes in Quantity of Water Soluble Nitrogen Compounds

Age of the seedling used	Length of hypocotyl (*cm.*)	Part of hypocotyl	Length (*cm.*)	NH_3-N per unit length (γ/*cm.*)	Glutamine amide-N per unit length (γ/*cm.*)	Asparagine amide-N per unit length (γ/*cm.*)	Amino-N per unit length (γ/*cm.*)	Water soluble-N per unit length (γ/*cm.*)	Soluble protein-N per unit length (γ/*cm.*)
3 day-old	11.6	Upper	1.8	5.07	1.30	0.00	60.8	76.7	8.15
		Middle	4.9	0.83	1.03	1.52	52.3	78.3	10.83
		Lower	4.9	0.83	0.44	5.95	76.5	106.7	13.90
6 day-old	19.7	Upper	9.0	0.92	0.08	2.60	31.6	50.8	2.73
		Middle	5.7	0.92	0.00	4.09	53.6	73.8	8.63
		Lower	5.0	1.35	0.60	3.17	61.1	79.5	9.56

Thus asparagine appears to migrate first from the cotyledon to the growing stem axis as the seeds begin to germinate, and, secondly, upwards in the axis concomitantly with the acropetal translocation of growth pattern. A similar migration of various metabolites, *e.g.* nucleic acid, sugar (*2, 7*), and acid soluble phosphate compounds (*5*), has already been reported ; and it has been pointed out that in the epigeal type of germination where the growth of epicotyl is preceded by that of hypocotyl, the hypocotyl would function as the secondary storehouse-organ for the epicotyl growth as the reserve nutriments of the cotyledon, the primary storehouse of the seed, are exhausted (*7*). As shown in next section asparagine can have a share in the nitrogen anabolism,

hence it would be a reasonable assumption that the amide may be transported from the tissues of either "primary" or "secondary" storehouse nature to the growing parts to contribute to the protein synthesis through the amino acid formation, and as the rate of protein synthesis drops there may occur a temporary accumulation of asparagine.

Only a little amount of glutamine was detected in every embryonic organ. It must be noted, however, that in the radicle the glutamine content, though small, was larger than that of asparagine. The ammonia level was very low in respective organs during the germination stage (Figs. 1–5). The low ammonia level would be due to the removal of ammonia by amide synthesis (see next section), glutamic dehydrogenase action (*4*), and other ammonia utilizing processes.

Amino Acid Formation from Asparagine and Keto Acid

The hypocotyls (70 g.) from the 3 day-old plants were homogenized by means of a Waring blendor for 3 minute with 70 ml. of $M/10$ phosphate-borate buffer (pH 6.5) ; squeezed through cotton cloth ; and centrifuged for 15 minutes at $1,100 \times g$. To the resultant supernatant ammonium sulphate was added to 0.3 saturation and the mixture was centrifuged for 15 minutes at $1,100 \times g$. The supernatant was mixed again with ammonium sulphate (0.7 saturation), and centrifuged for 20 minutes at $1,100 \times g$. The sediment obtained was suspended in 10 ml. of 0.03 M (pH 6.5) phosphate-borate buffer, and dialysed overnight against cold distilled water. The dialysate was centrifuged for 5 minutes at $800 \times g$. This final supernatant was employed in the following experiment.

As shown in Table II, when this preparation was incubated with asparagine and α-ketoglutarate, glutamate and ammonia were formed, asparagine amide-N being decreased. The addition of pyridoxamine and ATP caused a marked decrease in asparagine together with the corresponding increase in ammonia. In a separate experiment, the addition of pyridoxine and ATP, however, was found not to provoke such a decrease in asparagine. Pyridoxamine phosphate may be produced *in vivo* from pyridoxamine with the participation of ATP (*cf.* (*17*)). The experiments (Table II) also suggest that this coenzyme of transaminase is hardly separable from the apoenzyme by mere dialysis.

The above experiment suggests the occurrence of α-keto acid-dependent deamidation of asparagine in which transamination as well as deamidation are involved :

Asparagine $+ \alpha$-keto acid$\longrightarrow$oxaloacetic acid $+ \alpha$-amino acid $+ NH_3$.

TABLE II

Asparagine-α-Keto Acid Transaminase Action of Hypocotyl Preparation

Medium composition \ Content per 5 ml. medium	NH_3-N (γ)	Glutamine amide-N (γ)	Asparagine amide-N (γ)	Amino-N (*mg.*)	Amide and amino acid identified by paper chromatography
Preparation +asparagine	122	0	531	1.15	Asparagine
Preparation +asparagine +α-ketoglutarate	278	0	148	1.15	Asparagine Glutamic acid
Preparation +asparagine +α-ketoglutarate +pyridoxamine	501	0	93	1.17	Asparagine Glutamic acid
Preparation +asparagine +α-ketoglutarate +pyridoxamine +ATP+Mg^{++}	590	0	0	1.15	Asparagine (trace) Glutamic acid

Standard system—Hypocotyl preparation (see the text) 10 ml.; phosphate-borate buffer (*M*/10, pH 6.5) 1 ml.; α-ketoglutarate (*M*/10) 2 ml.; asparagine (*M*/10) 2 ml.*; (*M*/100) 0.5 ml.; pyridoxamine (*M*/80) 0.3 ml.; $MgCl_2$ (*M*/20) 0.2 ml. Final volume: 16 ml. Each flask was kept at 30° for 3 hours. Contents at the end of the incubation period are given.

* Asparagine equivalent to 870 γ amide-N per 5 ml. of medium was added.

Providing that in the above reaction asparaginase and aspartic-α-keto acid transaminase are involved, the preparation must produce aspartic acid, when it is incubated with asparagine alone and the action of the latter enzyme is blocked by some means. As the dialysis procedure in the extraction process may remove the whole keto acid originally contained in the tissues, aspartic-α-keto acid transaminase, if any, can't function there, so long as α-keto acid is not added. No aspartic acid production, however, is shown in system *Preparation+asparagine*, of Table II. It may, therefore, be concluded that asparaginase activity is negligibly small in the preparation used, and further, that in the present material asparagine-α-keto acid transaminase would be cooperating with deamidase as is shown in the Meister's scheme (*8*)

proposed for liver. However, with a paper chromatographic survey conducted with the first precipitate produced by the ammonium sulphate (0.3 saturation) treatment in the above extraction procedure, the present author has found that asparagine is decomposed to aspartic acid. Hence, asparaginase is considered to be also present in the intact hypocotyl. Both pathways of asparagine metabolism as illustrated in the Meister's scheme would be operative in hypocotyl.

The following fact shows the possibility that, *in vivo*, ammonia liberated by this α-keto acid dependent deamidation reaction can be utilized for the formation of amino acids. The water extract (100 ml.) was made from 100 hypoctyls excised from the 4 day-old plants. The addition of pyruvate or α-ketoglutarate to this extract was found to cause the decrease in both ammonia and asparagine and the increase in amino acid in the extract (see Table III).

By the way Table III shows an interesting fact that glutamine contained originally in the hypocotyl extract (*cf.* Fig. 2) was lost entirely during the incubation period, while in the presence of α-ketoglutarate added, glutamine was newly produced and/or prevented from decomposition in the incubation period.

TABLE III

Effects of α-Keto Acids on Amide Metablism in Hypocotyl

Medium composition \ Content per 10 ml. medium	NH_3-N (γ)	Glutamine amide-N (γ)	Asparagine amide-N (γ)	Amino-N (*mg.*)
Water extract alone	113	0	480	6.34
Water extract + pyruvate	54	0	216	6.87
Water extract + α-ketoglutarate	64	11	124	7.11
Water extract + α-ketoglutarate + ATP	48	11	167	7.11

Standard system—Water extract of hypocotyl (4 day-old) 20 ml.; phosphate-borate buffer (*M*/10, pH 6.5) 2.5 ml.; pyruvate or α-ketoglutarate (*M*/10) 2 ml.; ATP (*M*/100) 0.4 ml. Final volume: 25 ml. Each flask was kept at 30° for 3 hours. Contents at the end of the incubation period are given.

TABLE IV

Effect of Light on Amide Metabolism

Condition \ Content per 10 ml. hypocotyl extract	NH_3-N (γ)	Glutamine amide-N (γ)	Asparagine amide-N (γ)	Amino-N (*mg.*)
Light	16	5	216	1.08
Dark	21	5	264	0.97

See the text for experimental details.

Light was found to induce the same effect as the α-keto acid (Table IV). The 5 day-old etiolated plants were exposed to diffused sun light of about 200 lux. for 5 hours at 30°. Sixty ml. of the water extract were obtained from 30 hypocotyls (fresh weight 21 g.), and the contents of ammonia-N, glutamine amide-N, asparagine amide-N, and amino-N were compared with those of the control preparation made in the same way from the plants kept in the dark.

The contents of both ammonia and asparagine of the illuminated plants are lower than those of the control plants, whereas the amino acid content is higher in the illuminated plants. In the light conditions, therefore, ammonia and asparagine seem to be used in the amino acid formation. It is possible that light will promote the formation of α-keto acid through the photosynthetic mechanism. In this respect, it should be mentioned that the light-exposure is known to decrease asparagine in etiolated seedlings (*cf.* (*18*)).

The hypocotyl can produce asparagine from aspartate and ammonium salt (Table V). The hypocotyls of the 3 day-old plants were cut into discs of 3 mm. thickness with a razor blade. An aliquot of the discs was incubated with aspartate and ammonium salt for 3 hours at 30°. The hypocotyl discs were then homogenized together with the medium used, and the water extract was prepared to be assayed for asparagine formed. A positive result was obtained ; and it was further ascertained that the formation is promoted by the addition of ATP, in accordance with the McRary's description (*19*) that the formation of asparagine from ammonium aspartate (at pH 7.0) demands the energy supply of 3460 cal. The hypocotyl extract was revealed to have a far weaker ability of asparagine formation from asparatate and ammonium salt.

TABLE V

Synthesis of Asparagine from Ammonium and Aspartate

Medium composition \ Content per 10 g. (fresh weight) hypocotyl discs	NH_3-N (γ)	Glutamine amide-N (γ)	Asparagine amide-N (γ)
Hypocotyl discs alone	14	32	177
Hypocotyl discs +aspartate+NH_4^+	2112	38	744
Hypocotyl discs +aspartate+NH_4^++ATP	1544	50	864

Standard system—Hypocotyl discs (prepared from the 3 day-old seedlings) 15 g. (fresh weight); phosphate-borate buffer ($M/10$, pH 6.5) 10 ml.; aspartate ($M/10$) 3 ml.; ATP ($M/100$) 1 ml.; NH_4Cl (1 M) 1 ml. Final volume: 15 ml. Each flask was kept at 30° for 3 hours. Contents at the end of the incubation period are given.

SUMMARY

1. Changes in content of ammonia-N, amino-N, glutamine amide-N, asparagine amide-N, and total soluble-N of each seed organ of *Vigna sesquipedalis*, *i.e.* plumule, epicotyl, hypocotyl, radicle, and cotyledon were followed daily during the course of the germination stage.

2. Both amides and amino acids that disappeared from the cotyledon were apparently recovered as such in the growing tissues, in particular in the hypocotyl. The hypocotyl was able to produce asparagine newly from aspartate and ammonium salt; ATP added exerted an accelerating action on the synthesis.

3. From asparagine and α-ketoglutarate added, a partially purified hypocotyl preparation produced glutamic acid and ammonia. It was suggested that in this α-keto acid dependent deamidation of asparagine in hypocotyl the asparaginase-transaminase and/or the Meister's transaminase-deamidase may be involved. Pyridoxamine phosphate functioned as a cofactor in this reaction.

4. In the stem tissues a parallelism between the asparagine level and the protein synthesis was seen.

The author is greatly indebted to Assist. Prof. Dr. Y. Oota for his instructive

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STUDIES ON THE INHIBITION BY CARBON MONOXIDE AND NITROUS OXIDE OF ANAEROBIC NITROGEN FIXATION*

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The bulk of our present knowledge of biological nitrogen fixation has been acquired through studies following the biochemical activities of aerobic nitrogen fixers. Few investigations with anaerobic forms have been performed with success, presumably due to the difficulties encountered in maintaining abundant growths of these forms, and also to the sluggishness of their activities in fixing atmospheric nitrogen. In 1950, however, Rosenblum and Wilson (*1*) made a remarkable claim that with anaerobic forms (*Clostridium*) a level of nitrogen fixation far above that so far generally believed was realized, provided that their newly improved methods of experimentation were adopted. As for the mechanisms of the process, some workers (see (*2*)) have proposed entirely different schemes of reactions for anaerobic and aerobic fixations: they suggested that in aerobic fixation the combination of nitrogen with molecular oxygen is involved, a process evidently inconceivable in the cases of nitrogen fixation which proceeds under complete absence of molecular oxygen.

The authors mentioned above have also contributed to perform some fundamental studies, among others, the effect of molecular hydrogen on the anaerobic fixation, but no further approach has been made to disclose the nature of enzyme system (or systems) responsible for the process in question.

The present work was planned to elucidate the mode of some inhibitors, especially of carbon monoxide, and nitrous oxide, on the anaerobic nitrogen fixation. Based on the results obtained, it will be discussed that there is a certain features in common in the mechanism

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* A part of this study was reported before the Annual Meeting of the Botanical Society of Japan held on October 26, 1954 in Kyoto.

between anaerobic and aerobic fixations. Mentions will be also made on an improved culture method by which the bacterial cultures with most active nitrogen-fixing power can be obtained.

METHODS

The organism used throughout the present study was one selected from eight strains of the *Clostridium* which had been originally isolated from soil. A stock sand culture of the anaerobe was transferred into 15 ml. of the nitrogen-free medium (modified Winogradsky's solution: sucrose 20 g., $CaCO_3$ 10 g., K_2HPO_4 1 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., NaCl 0.01 g.; $MnCl_2$, $CuSO_4$, Na_2MoO_4, $Fe_2(SO_4)_3$ trace; water to 1000 ml.) and was heat shocked (*3*) for 10 minutes at 70° and incubated for about 48 hours at 30°. Two drops of the inoculation-culture thus obtained were added to 15 ml. of the potato extract medium (potato extract 500 ml., peptone 2 g., asparagine 0.5 g., sucrose 10 g., NaCl 2 g., $CaCO_3$ 10 g., water to 1000 ml.; pH 7.2) and incubated for further 20 to 24 hours at 30°. 0.5 ml. of the preculture thus obtained was inoculated into 14.5 ml. of the nitrogen-free medium in a 200 ml. shake flask equipped with rubber stopper, air in the gas phase was completely replaced with oxygen-free nitrogen in atmospheric pressure, and the flask was kept at 30° under constant shaking on a mechanical shaking device.

At the beginning and during the course of shaking culture, nitrogen was estimated on aliquots of 5 ml. of the culture, using the micro-Kjeldahl method. The quantity of fixed nitrogen was calculated by subtracting the initial nitrogen (about 30 μg./ml.) from total nitrogen found. Combined nitrogen was supplied as ammonium sulphate in a concentration of 280 μg. N/ml. and the uptake of ammonium-N was estimated by determining the content of unassimilated ammonium-N in 2 ml. of supernatant solution, direct distillation with the addition of concentrated alkaline solution being adopted.

Carbon monoxide was prepared, as usual, from formic acid and concentrated sulphuric acid, and nitrous oxide by heating the mixture of ammonium sulphate, potassium and sodium nitrate in a retort. Both gases were washed before use through two successive bottles of alkaline pyrogallol solution. Solutions of other chemicals used as inhibitors were sterilized separately by autoclaving, and 1 ml. each was added to 13.5 ml. of the testing medium.

RESULTS

Inhibition by Carbon Monoxide—In a preliminary experiment it was found that CO inhibited anaerobic nitrogen fixation even at appreciably lower partial pressures. With varying partial pressures of CO in the gas phase, its effect on N_2-fixation and uptake of ammonium-N was investigated. Typical results obtained are presented in Figs. 1 and 2. As may be seen, the N_2-fixation was appreciably affected by 0.1 per cent CO and almost completely inhibited by 1 per cent CO. The

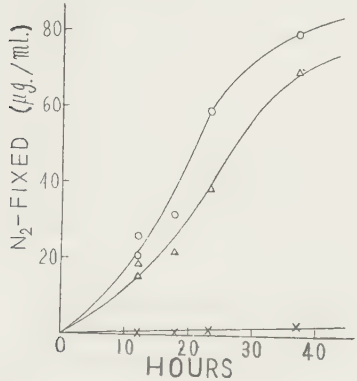

FIG. 1. Influence of carbon monoxide on N_2-fixation by *Clostridium.*
○: 100% N_2, △: 0.1% CO+99.9% N_2, ×: 1% CO+99% N_2.

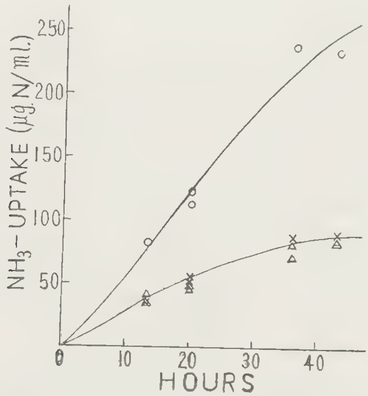

FIG. 2. Influence of carbon monoxide on uptake of NH_3-N by *Clostridium.*
○: 100% N_2, △: 1% CO+99% N_2, ×: 10% CO+90% N_2.

concentration for 50 per cent inhibition of anaerobic N_2-fixation seems to lie at about 0.3 to 0.7 per cent CO, values roughly similar to that previously reported by Lind and Wilson (*4*, *5*) for aerobic N_2-fixation by *Azotobacter.*

Uptake of ammonium-N by our bacterium was also inhibited in the presence of the gas but only to a less pronounced extent (e.g. 50

to 60 per cent inhibition by 1 per cent CO). The inhibition of ammonium-N uptake was not further increased above this level, even when the content of CO in the gas phase was raised as high as 100 per cent (Table I).

TABLE I

Effect of Carbon Monoxide on uptake of NH_3-N by Clostridium

CO-concentration %	0	0	0.1	1	10	100	100
Uptake of NH_3-N (*µg./ml.*)	129.8 (mean 122.6)	115.5	98.8	49.2	56.5	66.2	70.0
Inhibition %	—	—	19.5	59.8	53.9	46.1	43.0

The gas phase was complemented with N_2 to make up the total pressure of one atmosphere. Culture was shaken for 26 hours at 30°.

Inhibition by Nitrous Oxide—N_2O has been reported to exert a specific inhibitory action on aerobic N_2-fixation by *Azotobacter* (*6, 7*). The results obtained with our anaerobic organism are shown in Figs. 3 and 4. As may be seen from the figures, N_2-fixation was 70 to 80 per cent inhibited by 50 per cent N_2O, whereas no appreciable change in the uptake of ammonium-N was observed under the same condition. Even at the highest concentration studied (100 per cent N_2O) the gas caused only a slight inhibition of the process. The figures thus observed for the inhibitory effect of N_2O upon the anaerobic N_2-fixation are of the same order of magnitude as that reported for aerobic N_2-fixation by *Azotobacter* (*7*).

It may, therefore, be concluded that N_2O is a specific inhibitor of biological N_2-fixation, aerobic as well as anaerobic.

Other Inhibitors—Cyanide, azide, monoiodoacetate, *p*-chloromercuribenzoate and *o*-phenanthroline were also tested for their effects upon the anaerobic processes under investigation. The estimation of N_2-fixation was done at the end of 40 hours of culture. In this experiment, uptake of ammonium-N was measured by growing the bacteria in a 50 ml.-flask placed in an evacuated desiccator for the same period. From the results summarized in Table II it is evident that some of the inhibitors strongly influenced the N_2-fixation, but none of the effects observed seems to be specific for the fixation process *per se*. It ought to be mentioned here that none of these inhibitors have been considered

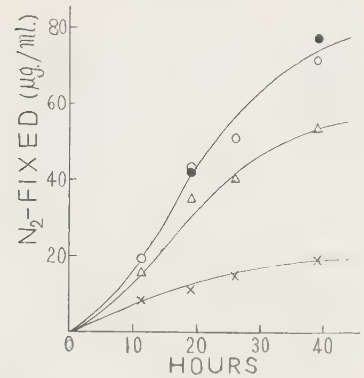

FIG. 3. Influence of nitrous oxide on N_2-fixation by *Clostridium*.
○: 100% N_2, ●: 50% Ar+50% N_2, △: 20% N_2O+80% N_2, ×: 50% N_2O+50% N_2.

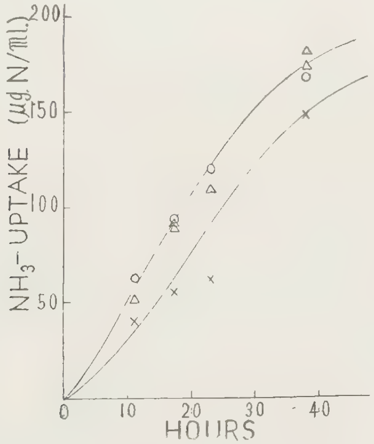

FIG. 4. Influence of nitrous oxide on uptake of NH_3-N by *Clostridium*.
○: 100% N_2, △: 50% N_2O+50% N_2, ×: 100% N_2O.

to be specific inhibitor of aerobic N_2-fixation.

Improvement of Culture Conditions—Typical course of anaerobic nitrogen fixation by the bacterium studied was exemplified by the curve (control, in 100 per cent N_2) in Figures 1 and 3, from which it may be seen that the amount of fixation was 60 and 55 μg./ml. in the first 24 hours, and 80 and 75 μg./ml. at the end of 40 hours of culture, in the

TABLE II

Percentage Inhibition Caused by Some Substances on N_2-Fixation and NH_3-Uptake by Clostridium

Inhibitor	N Source	Concentration of inhibitor (M)				
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
KCN	N_2	100	7			
	NH_3	100	0			
NaN_3	N_2		100	10		
	NH_3		100	12		
Monoiodoacetate	N_2	100	90	0		
	NH_3	100	88	5		
p-Chloromercuri-benzoate	N_2			100	70	0
	NH_3			100	90	0
o-Phenanthroline	N_2			100	0	
	NH_3			100	20	

Values given are percentage inhibition determined by comparison of the data with those of control experiments, in which the amount of fixation was 80 to 90 μg./ml. and uptake of NH_3-N was 180 to 200 μg./ml. Culture: 40 hours.

two series of experiment. Rosenblum and Wilson (*1*) obtained the value of 10 to 30 μg./ml. and 90 to 120 μg./ml., respectively, in the same time of incubation. In comparison with the values obtained by previous workers, the 40-hour's value obtained in the present experiment was lower, but the 24 hour's value was considerably higher. The lower level of the total yield of fixation in our experiments might be ascribed to the fact that the pH of the culture medium underwent a gradual fall during the growth of the bacterium to attain a final value as low as pH 5.4. In the experiments by the previous workers with *Cl. pasteurianum*, the shift in the reaction of the medium seemed to be less pronounced, a final pH value of pH 6.2 being recorded (*1*).

Three different measures were taken to maintain the pH of the medium as unaltered as possible: (1) the use of higher concentration of buffer solution, (2) the removal of CO_2 evolved during the culture and (3), neutralization by the use of ion exchange resin. In the first series, the dosis of K_2HPO_4 in the culture medium (see the description of the methods) was replaced by the addition of $M/20$ (end concentration) phosphate buffer of pH 7.5, $MgSO_4$ being sterilized separately

TABLE III

Effect of High Concentration of Buffer Solution, CO_2-Removal and Addition of Ion Exchange Resin on Anaerobic Nitrogen Fixation

Time (*hours*)	Amount of nitrogen fixed (*μg./ml.*)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Exp. 6
24	67.2 (5.6)	74.5 (5.8)	88.6 (6.0)	91.0 (5.8)		
	67.2 (5.6)	73.1 (5.6)	83.8 (5.8)	86.2 (5.8)		
48	86.7	96.2	96.2	111.7		
40					76.4	108.0
					79.0	110.1

Final pH values are listed in parenthesis.

Expt. 1 and Expt. 5: control series with nitrogen-free medium of standard composition described above (see page 776). Expt. 2: CO_2 removed from the medium by KOH. Expt. 3: with higher concentration of buffer solution. Expt. 4: combined effect of CO_2-removal and use of concentrated buffer. Expt. 6: with IRC-50.

to avoid the otherwise inevitable precipitation of the element. CO_2-removal was effected by inserting a small test tube containing a solution of KOH in the shake flask. In the last series of contrivance, the resin IRC-50 (in its Na-form) was used as a fixative for acidic substances produced, sterilization of the resin being previously effected with 70 per cent alcohol and ether. As may be seen from the results given in Table III, all of these methods proved to be effective in increasing the yield of N_2-fixation, although the final pH values of the medium were not so markedly ameliorated.

From the results of this set of experiments, it seems justified to claim that the anaerobic fixation of molecular nitrogen by *Clostridium* during the first 24 hours period can amount to 90 μg./ml., a level which is almost comparable to the values usually attainable, aerobically, with cultures of *Azotobacter*, which has been generally recognized to be the most powerful among various forms of nitrogen fixer.

DISCUSSION

In spite of the ubiquitous distribution and abundant growth in

soil, little weight has been laid to the role of *Clostridium* in the assimilation of atmospheric nitrogen in nature. The undue negligence of this class of organisms has been based primarily on the fact that in ordinary laboratory experiments, the rate of anaerobic fixation (usually 20 to 30 μg./ml. in 7 to 10 days) was far slower than that of aerobic fixation performed by *Azotobacter*. The results of our experiments are in accord with those of Rosenblum and Wilson, who claimed that the sluggishness of anaerobic fixation had been in fact due to the inadequate experimental method for the estimation of anaerobic N_2-assimilation ; it was realized in the present experiments that the rate of fixation by *Clostridium* is almost comparable with those by *Azotobacter* in an appropriate condition. From our results it seems justified to state that in order to make the fixation proceed steadily for a considerable period of incubation, measures should be taken to avoid the gradual fall of the pH values of the medium. The use of sufficiently effective buffer solution, elimination of acidic products of fermentation (CO_2 and, presumably, organic acids) from the reaction system, will do equally well for the purpose.

The sensitivity of aerobic N_2-fixation to carbon monoxide has been the subject of repeated investigations by various workers. The inhibition of the process in question by the gas, generally recognized as a respiratory poison, lead them to the concept that the process involves the oxidation of molecular nitrogen participated with some kind of hemoprotein, as the first step of the reaction (*8*). The idea of a combination of N_2 with O_2 as suggested by other workers (*2*, *9*) is also a result of consideration along the same line of arguments.

Our findings in the present work, that CO affects the anaerobic N_2-fixation (*Clostridium*), seem to be of certain importance, especially from two angles of considerations. In the first place, it is indicative of the situation that the sensitivity to CO alone does not necessarily provide a conclusive ground for reasoning of the direct involvement of molecular oxygen, nor of hemoprotein system ; although, indeed, in most of the biological processes known to be CO-sensitive, the actual participation of molecular oxygen seems to have been established. In the second place, the inhibition of either aerobic and anaerobic process by CO, and the similarity in their sensitiveness to the gas (see above, page 777), seems to suggest the possibility of a mechanism in common in the two processes in question.

Inhibitory effect of CO on N_2-fixation is much stronger than that

generally observed on the other biological processes, e.g. respiration. In this connection the elaborate work of Kempner and Kubowitz (*10*) on the CO-inhibition of butyric fermentation by *Clostridium butyricum* would be worth mentioning. In this case, a 50 per cent inhibition was observed at a partial pressure of 0.015 atmosphere (at 8° and pH 6.6).

The phenomenon of inhibition by N_2O, which also points to the similarity in the behaviour of aerobic and anaerobic N_2-fixers, seems to provide us with another clue in understanding the mechanism of the process in problem. The insensibility of assimilation of ammonium-N by *Clostridium* is in striking contrast to the pronounced susceptibility of N_2-fixation system and seems to promise the utility of this specific poison in the further analysis of the processes concerned.

Addendum: Recently the writer found two articles in *Chemical Abstracts* summarizing the papers of Virtanen and his co-workers about the inhibitory effects of CO (*11*) and N_2O (*12*), respectively, on anaerobic nitrogen fixation. The results referred to in the abstracts almost coincided with ours reported in the present paper, though the level of N_2-fixation attained by the Finnish investigators seem to be much lower than that in our experiments. At the present, the writer has not been favoured with the opportunity to know if they have altered their former opinion as to the mechanism of anaerobic N_2-fixation.

SUMMARY

1. With the cultures of a strain of *Clostridium* isolated from soil, the process of anaerobic fixation of molecular nitrogen as well as its inhibition by various substances was studied.

2. For the anaerobic N_2-fixation to be maintained in its active condition during sufficiently long culture periods, cares must be taken to keep the pH of the medium as constant as possible.

3. The level of anaerobic N_2-fixation thus achieved was usually as high as 90 μg. N per ml. of the culture solution during the first 24 hours' incubation, a value almost comparable with those obtained by previous workers in aerobic fixation with *Azotobacter*.

4. Carbon monoxide and nitrous oxide were found to inhibit the anaerobic N_2-fixation in a specific manner, in that they do not affect (N_2O) or do inhibit only in less pronounced extent (CO) the assimilation of ammonium-N by the bacterium.

5. Cyanide, azide, monoiodoacetate, *p*-chloromercuribenzoate and *o*-phenanthroline were also found to be powerful inhibitors of the

anaerobic process, though their action on the fixation system seemed to be unspecific in nature.

6. Based on these findings, some discussions were made on the mechanism of the biological N_2-fixations.

The writer wishes to express his sincere gratitude to Prof. T. Mori of this laboratory for his continuous encouragement and advices. Thanks are also due to Prof. H. Tamiya of the University of Tokyo and Dr. A. Takamiya of the Tokyo Institute of Technology for their valuable suggestions. The work was partly supported by the Grant in Aid for Scientific Research from the Ministry of Education.

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The Journal of Biochemistry, Vol. 42, No. 6, 1955

THE FORMATION OF SUCCINATE BY MYCOBACTERIUM TUBERCULOSIS AVIUM EXTRACTS[1]

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(Received for publication, September 5, 1955)

Previously, all the enzymes involved in the tricarboxylic acid (TCA) cycle were extracted in cell-free state from *Mycobacterium avium* (*2*, *3*), and it was concluded that TCA cycle was operating mainly in the terminal respiration of this organism.

More recently, in the course of quantitative measurement of the members of TCA cycle, it was noted that succinate was strikingly formed from L-malate not *via* TCA cycle (*4*).

In the present paper, the evidence and mechanism of the formation of succinate in the cell-free extract of *M. avium* will be described.

EXPERIMENTAL

Methods

Preparation of Cell-free Extract—*Mycobacterium avium*, strain Takeo, was employed. The organism was grown on glycerol-bouillon medium for 4 days according to the previous report (*3*). About 20 g. of washed cells were ground with alumina plus a small amount of distilled water for 40 minutes in a chilled-mortar, and then mixed with 50 ml. of distilled water. After 60 minutes, the mixture was centrifuged on 12,000 r.p.m. for 30 minutes, and the obtained supernatant fluid was used immediately as the enzyme preparation, or stored in the frozen-state for one week with little loss of activity untill needed. Such cell-free extracts are able to oxidize rapidly all the members of TCA cycle, with the exception of succinate which is oxidized only slowly in the absence of glutamate as reported earlier (*2*, *3*).

Determination—Reactions were all carried out in Warburg vessels at 30°. Citrate, α-ketoglutarate, succinate, L-malate, oxalacetate and pyruvate were estimated according to the methods of Natelson *et al.* (*5*), Krebs (*6*), Krebs (*7*), Nossal (*8*), Edson (*9*) and Warburg *et al.* (*10*) respectively.

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1) A preliminary report of this work was presented at the 1st Meeting of Kinki District of Japanese Biochemical Society, December, 1953 (*1*).

RESULTS

Formation of Succinate from L-Malate not via TCA Cycle—Table I shows the simultaneous formation of citrate, α-ketoglutarate and succinate

TABLE I

Formation of Citrate, α-Ketoglutarate and Succinate from L-Malate by the Cell-free Extract of M. avium

Reaction time	Formation			Oxygen uptake	Calculated oxygen uptake§
	Citrate	α-Ketoglutarate	Succinate		
min. 20	1.7	1.8	6.4	6.3	22.6
60	5.8	2.3	17.6	19.8	57.3

All values in micromoles. The reaction mixtures contained 100 μM of L-malate, 160 μM of phosphate buffer (pH 6.0) and 2 ml. of the cell-free extract per 4 ml. of total volume. The reaction was carried out at 30° in the air in Warburg vessel.

§ If these acids are formed exclusively by way of TCA cycle using molecular oxygen as electron acceptor, three, four, and five oxygen of atoms are needed for the production of one mole of citrate, α-ketoglutarate and succinate from two moles of L-malate respectively.

from L-malate in the cell-free extract. The rapid appearance of these acids gives a strong evidence supporting that TCA cycle is actually operating in this extract. However, the remarkable accumulation of succinate cannot be fully explained in terms of the oxidative TCA cycle alone. For example, 22.6 μM of oxygen uptake will be needed to produce these acids from L-malate for 20 minutes in the experiment of Table I, if the reaction proceeds exculsively by way of TCA cycle and molecular oxygen is utilized as terminal electron acceptor, but only 6.3 μM of oxygen was actually shown to be utilized. This fact suggests the occurrence of one or more pathways besides TCA cyle.

Moreover, even under anaerobic conditions, marked formation of succinate from L-malate was found as well as aerobically, as shown in Experiment 1 of Table II. On the other hand, no detectable amount of citrate was formed anaerobically.

Accordingly, it is apparent that the mechanism of the formation of succinate and that of citrate from L-malate are quite independent each other.

TABLE II

Comparison between L-*Malate and Oxalacetate for the Formation of Citrate and Succinate*

Substrate	Reaction time	Gas phase	Formation	
			Citrate	Succinate
	min.		μM.	μM
L-Malate 150 μM	80	Air	14.1	18.0
”	”	100% N_2	0.4	22.0
Oxalacetate 80 μM	60	Air	24.1	2.6
”	”	100% N_2	1.3	4.0

Experimental conditions were the same as in Table I except substrates or gas phase.

Further, oxalacetate was examined as substrate in place of L-malate. Table II (Experiment 2) indicates that citrate was formed remarkably from oxalacetate as well as L-malate under aerobic conditions, whereas succinate was formed only slightly from oxalacetate either aerobically or anaerobically. Thus, it seems very probable that the formation of succinate from L-malate does not pass through oxalacetate.

These findings strongly suggest that succinate is formed reductively from L-malate. The following experiments were all performed under anaerobic conditions. As, in this case, evolution of carbon dioxide from L-malate was observed and sometimes a small amount of pyruvate was detected (Table III), it was suggested that these substances were end-products of the oxidation coupled with the reduction of L-malate to succinate. However, the recovery of pyruvate formed was too small to give such evidence. But it became soon clear that pyruvate was dissimilated in this extract even anaerobically as shown in Table IV. It therefore appeared that the pyruvate produced from L-malate was rapidly metabolized. But, which was a direct oxidation product pyruvate or oxalacetate was left to be decided, since the extract was capable of decarboxylating oxalacetate to pyruvate as described previously (*2, 3*). Therefore, the experiment was conducted in the presence of excess semicarbazide. Table III (Experiment 3) shows that in this case significant amounts of oxalacetate accumulate, while no carbon dioxide evolves.

Moreover, in the presence of semicarbazide, the ratio of L-malate consumed to succinate formed was shown to be usually about 2.0 (Table

V). From these results, it is considered that the primary step of the conversion of L-malate is the coupling of its oxidation to oxalacetate with its reduction to succinate. As the behaviour of fumarate is found to be essentially the same as L-malate, and the extract contains active fumarase, the following scheme may be presented.

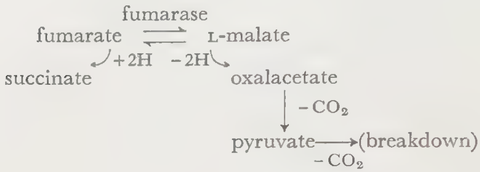

TABLE III

Anaerobic Formation of Carbon Dioxide, Pyruvate and Oxalacetate from L-Malate

Expt. No.	Addition	Formation			
		Succinate	Carbon dioxide	Pyruvate	Oxalacetate
1	None	14.8	15.2		
2	"		22.4	5.7	0.0
3	"		9.2		0.0
	Semicarbazide 200 μM		0.0		19.9

All values in micromoles. Each vessel contains 100 μM of L-malate. The reactions were carried out in 100 per cent N_2.

TABLE IV

Anaerobic Breakdown of Pyruvate

Expt. No.	Consumption	Formation	
	Pyruvate	Carbon dioxide	Volatile acids
1	40.0	19.1	10.1
2		21.0	28.4

All values in micromoles. Each vessel contained 50 μM of pyruvate, 100 μM of phosphate buffer (pH 6.0), 10 μM of $MnCl_2$ and 3 ml. of the extract. The reaction was carried out in 95 per cent N_2 and 5 per cent CO_2.

TABLE V

Formation of Succinate and Disappearance of L-Malate

	Reaction time (*minutes*)				
	120	180	240	300	450
Disappearance of L-malate (1)*	31.6	46.0	50.4	57.7	67.2
Formation of succinate (2)	13.6	26.0	30.0	31.8	33.0
(1)/(2)	2.4	1.8	1.7	1.8	2.0

All values in micromoles except the value of (1)/(2). Each vessel contained 85 μM of L-malate, 200 μM of semicarbazide (pH 6.5), 150 μM of phosphate buffer (pH 6.5) and 4 ml. of the extract. The reaction was carried out in 100 per cent N_2.

* These values do not contain the amounts of fumarate formed from L-malate by fumarase in the extract.

Properties of the Coupled Oxido-Reduction System—The properties of this coupled oxido-reduction system was studied in the presence of excess semicarbazide in order to block the subsequent processes. This reaction is not inhibited by 2,3-dimercaptopropanol (BAL), cyanide, iodoacetate, fluoride and malonate. However, malonate inhibits the aerobic oxidation of succinate catalyzed by the same preparation (Fig. 1). Either semicarbazide or hydroxylamine stimulates rather than inhibits the rate

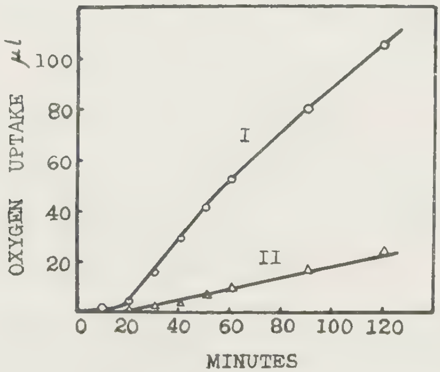

FIG. 1. Effect of malonate on the aerobic oxidation of succinate by the extract.

Curve I: 50 μM succinate, 100 μM glutamate, 140 μM phosphate buffer (pH 6.0) and 4 ml. extract. Curve II: the same as in Curve I except 300 μM malonate.

of the coupled oxido-reduction. Redox dye such as methylene blue, nile blue or janus green gives no stimulation.

The treatment of the extract with active charcoal reduces the reaction rate to one half of the original value, and the addition of the boiled untreated extract restores partially the activity (Table VI).

TABLE VI

Effect of Treating with Charcoal

Preparation	Addition	L-Malate consumption
Original extract	None	53.6
Charcoal treated extract	"	28.0
"	DPN	27.3
"	Boiled original extract	39.0

All values in micromoles. Each vessel contained 160 μM of L-malate, 200 μM of semicarbazide, 160 μM of phosphate buffer (pH 6.0) and extract. The reaction was carried out in 100 per cent N_2. The activity of coupled oxido-reduction was expressed as the consumption of L-malate for 3 hours.

Diphosphopyridine nucleotide (DPN), flavinadenine dinucleotide (FAD), riboflavinphosphate (FMN), adenosinetriphosphate (ATP), Mg^{++}, Mn^{++} and Fe^{++} are unable to replace the boiled extract.

DISCUSSION

In 1937, Dewan and Green (*11*) found the occurrence of the dismutation of fumarate (or L-malate) in the heart muscle preparation. The over-all reaction was the same as described in this paper. Since the rate of the reaction was very extremely slow, Slater (*12*) concluded that whether it was of great quantitative importance *in vivo* was doubtful.

However, this reaction was shown to occur relatively fast in our bacterial extract even under aerobic conditions (Table I). Therefore, it is suggested that it plays a rôle in the metabolism of *Mycobacterium avium*.

According to Slater (*13*), it is believed that the reduction of fumarate by L-malate in the animal tissue proceeds as follows:

$$\text{L-Malate} \xrightarrow[\text{dehydrogenase}]{\text{L-malic}} \text{DPN} \xrightarrow[\text{protein}]{\text{flavo-}} \underset{\text{factor}}{\text{Slater's}} \longrightarrow \text{cytochrome b} \xrightarrow[\text{dehydrogenase}]{\text{succinic}} \text{fumarate}$$

The nature and the number of enzymes involved in the over-all reaction in *M. avium* extract have yet to be elucidated. The latest work in our laboratory, indicated that L-malic dehydrogenase operates in our system too (4)[2], but little information is available about the enzyme responsible to the reduction of fumarate (or its derivative). The participation of succinic dehydrogenase is unlikely from the point of view that the coupled oxido-reduction system is malonate-insensitive. Furthermore, it must also be stressed that the rate of this reaction involving the reduction of fumarate is much more rapid than the oxidation of succinate. As an example, under the experimental conditions as illustrated in Fig. 1, about 3.5 μM of succinate was aerobically oxidized for 90 minutes. On the other hand, the same extract catalyzed the formation of 16 μM of succinate from L-malate in the presence of semicarbazide for 90 minutes anaerobically.

Thus the latter reaction proceeds about 4.6 fold faster than the former reaction. It cannot be easily accepted that succinic dehydrogenase catalyzes the reverse reaction, *e.g.*, the reduction of fumarate more rapidly.

Fractionation and reconstruction of the enzyme components are necessary to elucidate the more detailed mechanism and physiological significance of this reaction, and are now being investigated.

SUMMARY

The alumina ground extract of *Mycobacterium avium* catalyzes the coupled oxido-reduction as the following equation.

2 L-Malate ⟶ oxalacetate + succinate
(or fumarate)

This reaction is inhibited by neither malonate nor BAL. It is suggested that this reaction plays a rôle in the metabolism of *M. avium*, since it proceeds considerably fast.

The authors wish to express their thanks to Prof. S. Watanabe, the chief of the Toneyama Institute for Tuberculosis, and Prof. S. Akabori, Faculty of Science, Osaka University, for their counsels and encouragements during this investigation.

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STUDIES ON SWEET POTATO PHOSPHATASE

I. SPECIFICITY

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(Received for publication, September 5, 1955)

The separation and the partial purification of an acid phosphatase from sweet potato tubers was reported in the previous paper (*1*). This phosphatase might be classified as *Pyrophosphatase II* according to the Sumner-Somers' nomenclature (*2*). In the course of the purification of this enzyme, however, no indication was obtained of the separation of an enzyme specific for any of adenosine triphosphate (ATP), inorganic pyrophosphate or phosphomonoesters used as substrates. In the present paper, further attempts to separate specific phosphatases and some kinetic studies on the action of the purified enzyme preparation upon the several substrates are described.

In recent years, strictly specific phosphatases have been isolated (*3–8*). On the other hand, it has been also suggested that there is such a single phosphatase that can hydrolyze various types of phosphate linkages (*9–12*).

The results in this paper suggest that our preparation of sweet potato phosphatase is one of the latters, that is, a non-specific enzyme.

MATERIAL AND METHODS

Preparation of Enzyme—Acid phosphatase was purified from extracts of sweet potato (*Norin* I, produced in Chibaken, Japan) tubers according to the method described previously (*1*), which included fractional precipitations with acetone (40–60 per cent) and with $(NH_4)_2SO_4$ (0.40–0.65 saturation), pH 4 treatment and alumina $C\gamma$ adsorption. This preparation was used in further attempts to separate enzymes and for kinetic studies of the phosphatase reaction.

Substrates—The following compounds were used as substrates of enzymatic action : Synthetic Na-diphenylphosphate ; Na-β-glycerophosphate (Kanto) ; inorganic Na-pyrophosphate (Kanto) ; ATP prepared from rabbit muscle, 85 per cent purity ; Na-metaphosphate, prepared according to the method of G. Von Knorre (*13*), which was shown to contain 60 per cent trimetaphosphate and 40 per cent hexametaphosphate

by the analytical method of Jones (*14*) ; synthetic Na-monophenylphosphate.

Measurement of Phosphatase Activity—Unless otherwise noted, the reaction mixture consisted of 2 ml. of 0.1 *M* citrate buffer of pH 5.5, 1 ml. of 0.01 *M* substrate solution, 1 ml. of H_2O or other additions, and 1 ml. of enzyme solution. During incubation at 30°, aliquots of the reaction mixture were withdrawn at appropriate times when extent of hydrolysis was limited to several per cent. Determinations of inorganic orthophosphate or phenol were made with the aliquots. In the kinetic studies, a best caution is necessary to avoid confusion caused by the inhibition of enzymatic action by orthophosphate. 0.1 to 2 μg. of orthophosphate were measured colorimetrically with a slight modification of the method of Youngburg and Youngburg (*15*). When pyrophosphate or arsenate were present, the method of Fiske and Subbarow (*16*) was used, because, with the former method, these compounds give the same color as does orthophosphate. Phenol was determined with the method of Folin and Ciocalteu (*17*).

Chromatography—Corn starch was packed in a 15 cm. glass tube of 1 cm.-diameter and was washed with dilute HCl, water and acetate buffer of pH 4.0, successively. The pH of the purified phosphatase to be chromatographed was adjusted to 4.0. Protein adsorbed on the upper part of the column was eluted with 0.1 *M* citrate buffer of pH 6.7. The effluent was collected in test tubes in 2 ml. portions. The flow-rate was about 1 ml. per hour. The protein and phosphatase contents of each tube were measured.

RESULTS

Purification of Enzyme—The preparation of sweet potato phosphatase was further subjected to fractional precipitations with ethanol and with $(NH_4)_2SO_4$. The specific activity of the final preparation was 500-fold as high as that of the original extract, amounting to 250,000 as Qp (for 0.02 *M* β-glycerophosphate at pH 5.5, 30°). But, the comparison of the activities upon phosphomonoesters, inorganic pyrophosphate and ATP showed that there had been no obvious separation of specific enzymes in the course of the purification. On the other hand, it was observed that the specific activity upon metaphosphate had increased much more than that upon the other phosphates in the course of the purification, especially at the step of acetone treatment.

Filter Paper Ionophoresis and Starch Column Chromatography—Filter paper ionophoresis with citrate buffer or phosphate buffer of various pH's were carried out upon the purified enzyme. Separation of phosphatase was not observed. Fig. 1 shows typical data on the distribution of the activities among the effluents from starch column chromatography. Two peaks of the activities were observed. The result was reproducible, although it is not understood what the separation into these peaks means. With

regard to optimum pH, the effects of addition of Ca^{++} and Mg^{++} on the activities, and to the ratios of the activities upon the various substrates, evident differences were not observed between the fractions represented by these peaks. In all the fractions, ratios of the activities upon β-glycerophosphate, pyrophosphate and ATP scarcely changed, while, in several tubes, the activity upon metaphosphate exceeded evidently those upon the other substrates.

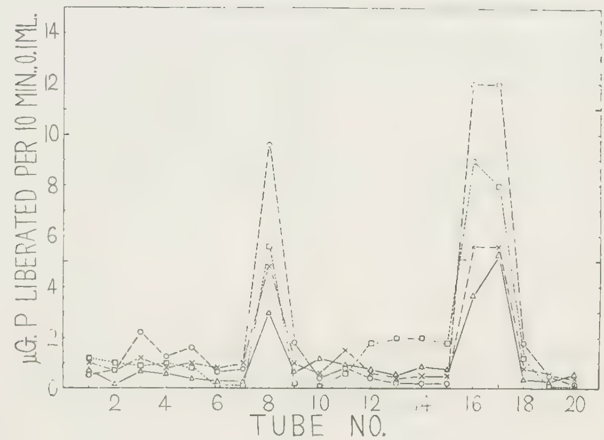

Fig. 1. Starch column chromatography.

The purified phosphatase solution was adsorbed on a starch column at pH 4.0 with acetate buffer and eluted with 0.1 *M* citrate buffer of pH 6.7. Each tube contained a 2 ml. portion of effluent. For details, see text.

—△—, β-glycerophosphate ; ---×---, ATP ; --○--, pyrophosphate ; ----□----, metaphosphate.

Influence of Heating at Various pH's—Enzyme solutions adjusted to various pH's with citrate buffer were held for 24 hours at 37°, for 3 hours at 50°, or for 1 hour at 60°, then their activities with each substrate were measured at pH 5.5. The per cents of the original activities remaining are given in Fig. 2. The residual activities varied with the pH values used and with the particular substrate tested, but for all the substrates, the highest activities were observed at pH 5.5 to 6.0. Analogous results were obtained with ATP as a substrate. Dilute enzyme solutions were more readily inactivated by heat than concentrated ones were.

Mutual Inhibition by Substrates—When a single enzyme reacts with two substrates simultaneously, and reacts with each of them according to the scheme of Briggs and Haldane (*18*), and if the substrates are assumed to attach competitively to the same or the neighbouring points on the enzyme, then the combined velocity can usually be calculated by substituting the values of v_1, v_2, K_{m1}, and K_{m2} in the following equation,

$$v = \frac{v_1(S_1+K_{m1})}{S_1+K_{m1}(1+S_2/K_{m2})} + \frac{v_2(S_2+K_{m2})}{S_2+K_{m2}(1+S_1/{}_{m1})} \qquad (1)$$

where, v_1 and v_2 are the reaction rates of substrates 1 and 2 respectively in absence of the other substrates, S_1 and S_2 are the concentrations of the

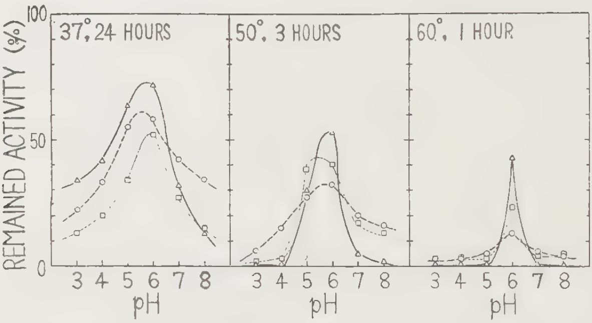

FIG. 2. Heat inactivation.

The enzyme solutions in 0.05 *M* citrate buffer of various pH's were held for 24 hrs. at 37°, for 3 hrs. at 50°, or for 1 hour. at 60°. Immediately after the above times, activities were measured at pH 5.5 in the standard test. The residual activities are expressed in per cent of the original.

—△—, β-glycerophosphate ; --○--, pyrophosphate ; ---□---, metaphosphate.

substrates, and K_{m1} and K_{m2} Michaelis constants for the substrates, respectively. In the present experiment, two of the substrates were incubated together with enzyme and the rate of liberation of orthophosphate was measured. In Table I, the values of $v_{obs.}$ thus obtained are compared with the values of $v_{calc.}$ obtained from equation 1. The values of v_1, v_2, K_{m1} and K_{m2} were determined spearately in other experiments with a single substrate. Close agreement is shown except in systems where metaphosphate is one of the components.

TABLE I

Reaction Rate with Mixtures of Substrates

The concentration of each substrate was 1/1500 *M*.

Substrates	$v_{obs.}$	$v_{calc.}$
Pyrophosphate+phenylphosphate	31	31
Pyrophosphate+ATP	57	52
Pyrophosphate+metaphosphate	53	40
Pyrophosphate+β-glycerophosphate	65	64
Phenylphosphate+ATP	66	60
Phenylphosphate+β-glycerophosphate	39	40
Phenylphosphate+metaphosphate	61	49

Inhibition of Phenylphosphatase Reaction by the Other Substrates—For competitive inhibition, Hunter and Downs (*19*) had introduced the equation,

$$I\frac{\alpha}{1-\alpha}=K_i+\frac{K_i}{K_s} \qquad (2)$$

where, the fractional activity, α, is equal to v_i/v (v_i and v are the reaction velocity in presence of an inhibitor or another substrate and that in its absence, respectively), K_s is Michaelis constant for the substrate, K_i is Michaelis constant for the inhibiting substrate or the dissociation constant of the enzyme-inhibitor complex assumed, I is the concentration of the inhibitor or of the inhibiting substrate, and S is that of the substrate.

If an enzymatic reaction with one substrate obeys this equation in the presence of an inhibitor or of another substrate, the plot of $I\cdot\alpha/(1-\alpha)$ against S should give a straight line, its intercept on the $I\cdot\alpha/(1-\alpha)$axis being equal to K_i, and that on the S axis to $-K_s$. If it is assumed that the substrate and the inhibiting substrate are reacting with a single enzyme and that they attach to the same site on the enzyme to form the enzyme-substrate complex, the K_i of the inhibiting substrate evaluated by this process will be equal to K_m, Michaelis constant of the inhibiting substrate determined separately. As an example, enzymatic hydrolysis of phenylphosphate in the presence of the other substrates was studied by measuring the liberation of phenol. The results are shown in Table II and Fig. 3. Except for the case of metaphosphate, close agreement is seen between the K_m values and the K_i values in Table II. The straight lines in Fig. 3 show the theoretical ones which cross abscissa at

TABLE II

Inhitition of Phenylphosphatese Reaction by Other Phosphates

K_m was determined by Lineweaver and Burk plot (*20*) of the reaction in which 1/4000 to 1/250 *M* of each substrate was incubated at pH 5.5 and 30° with sweet potato phosphatase. K_i is the mean value calculated from Eq. 2.

Compounds	K_m determined in the single-substrate system	K_i determined by inhibition against phenylphosphate
	M	*M*
Phenylphosphate	6.0×10^{-4}	—
Orthophosphate	—	2.0×10^{-4}
Diphenylphosphate*	inactive	ineffective
β-Glycerophosphate	20.0×10^{-4}	20.0×10^{-4}
Pyrophosphate	3.1×10^{-4}	3.3×10^{-4}
ATP	6.7×10^{-4}	6.7×10^{-4}
Metaphosphate	8.0×10^{-4}	32.0×10^{-4}
Phenol*	—	ineffective
Glycerol*	—	ineffective

* Concentration up to 1×10^{-2} *M* was tested.

the position corresponding to the $-K_s$ value for phenylphosphate and cross ordinate at the positions corresponding to the K_m values for the other substrates. The extent of inhibition by metaphosphate is far from the expected value, while, for the other inhibiting substrates, the observed values are close to the theoretical lines. Diphenylphosphate was not hydrolyzed by this enzyme preparation, and did not affect the enzymatic reaction with the other substrates.

Inhibition by Arsenate and Borate—The initial reaction velocity was measured for varied concentrations of substrates in presence of 2×10^{-2} *M* arsenate or of 2×10^{-3} *M* borate and was compared with that in their absence. The results for arsenate are shown in Fig. 4 and for borate in Fig. 5. Plots of $I \cdot \alpha/(1-\alpha)$ against S give straight lines, which intercept ordinate at the same point independently of the particular substrate. The K_i value was 4.9×10^{-4} *M* for arsenate and 8.3×10^{-3} *M* for borate, with the exception of the case when metaphosphate was used as substrate. In this case, 1.6×10^{-4} *M* and 3.0×10^{-3} *M* were obtained as the K_i values for arsenate and borate respectively.

Influence of Other Compounds—Compounds found generally to affect

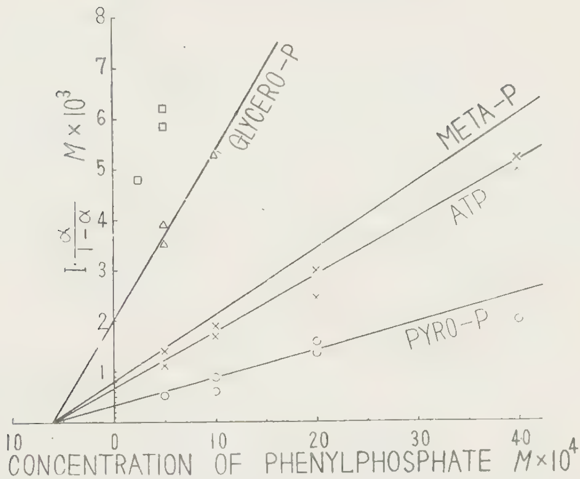

FIG. 3. Inhibition of phenylphosphatase reaction by the other substrates.

The straight lines are theoretical, their intercept on abscissa being equal to $-K_m$ value for phenylphosphate and those on ordinate to K_m values for the other substrates. Phenylphosphate was incubated with sweet potato phosphatase at 30°, pH 5.5 in absence of and in presence of 6.6×10^{-4} *M* or 2×10^{-3} *M* of β-glycerophosphate (△), pyrophosphate (○), ATP (×), or metaphosphate (□), and the initial rate of liberation of phenol was measured.

phosphatase activity were tested with our enzyme preparation for several substrates. Some of the results are listed in Table III. It is seen that with the exception of NaF, Na_2MoO_4, cysteine and KCN, the compounds are noncompetitive inhibitors and inhibit the reactions of our phosphatase preparation to about the same extents with all substrates. NaF and Na_2MoO_4 were proved to be competitive inhibitors. On the other hand, cysteine and KCN showed some activating effect.

DISCUSSION AND CONCLUSION

The specific activity for metaphosphate increased exceedingly over that for the other substrates during the purification, but this does not necessarily mean the presence of a specific metaphosphatase. In this respect, an experiment on heating may be mentioned. Table IV shows the change in K_m caused by heating our enzyme preparation. K_m for

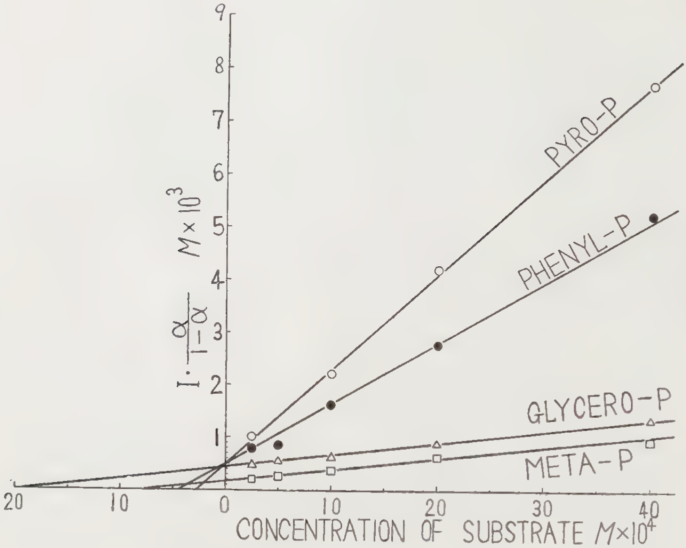

FIG. 4. Inhibition by arsenate.

Varied concentrations of the substrates were incubated with sweet potato phosphatase at 30° and pH 5.5 in presence of 2×10^{-4} *M* of arsenate. The initial rates of hydrolysis were measured by estimating the liberation of orthophosphate with the method of Fiske and Subbarow (*16*), and were compared with those in absence of arsenate.

Substrates : β-glycerophosphate (△), phenylphosphate (●), pyrophosphate (○), metaphosphate (□).

β-glycerophosphate decreased upon heating, while that for pyrophosphate increased. It is suggested that heating does not cause an all-or-none inactivation, but rather, alters the properties. So, changes in the ratio of the activities upon different substrates during heating or other treatments do not immediately prove the existence of two or more different enzymes. Rather, the identity of the most favourable pH for stability, and the uniform behavious of phosphatase activities during the treatments in purification and chromatography suggest that the separation of enzymes specific for one of the substrates may be almost impossible. The possible presence of specific enzymes can be discussed on the basis of the kinetic studies. As far as the present experiments on competitive inhibition show, metaphosphatase activity must be distinguished from the activities upon the other substrates used. However, metaphosphatase activity was

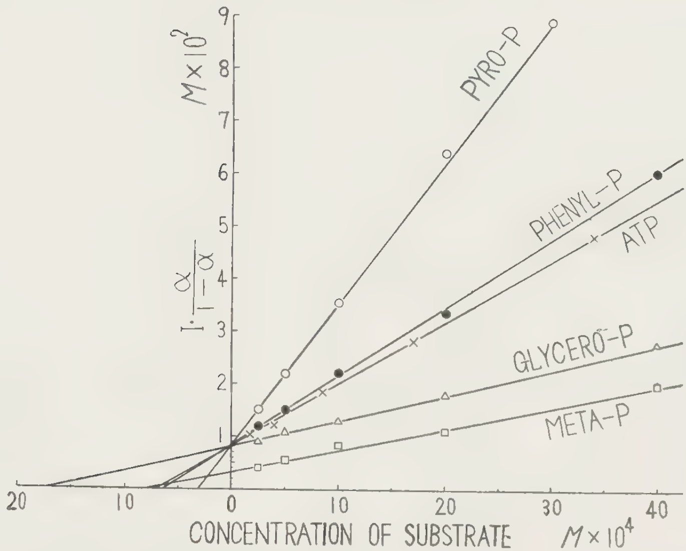

FIG. 5. Inhibition by borate. 2×10^{-3} *M* borate was added. Others are the same as in Fig. 4.

Sugstrates : β-glycerophosphate (△), phenylphosphate (●), pyrophosphate (○), ATP (×), metaphosphate (□).

determined by estimating the liberation of orthophosphate, and presumably the liberation of orthophosphate is not the first step of hydrolysis of metaphosphate. On this account, a more detailed analysis is required to make a precise conclusion. On the other hand, the activity upon phosphomonoesters could not be distinguished from that upon pyrophosphate or ATP with respect to the nature of the combination between the enzyme and the substrate or the inhibitor. Although a more direct experimental evidence is necessary, it may now be suggested that phosphomonoesters, pyrophosphate and ATP attach to the same point on a single enzyme in the present preparation. If this is not so, any specific enzymes present resemble each other so closely as protein and in the nature of the sites which participate directly in the enzymatic action that their separation will be very difficult. The assumed non-specific phosphatase seems to combine with H_3PO_4, H_3BO_4 and H_3AsO_4 as well as with the terminal phosphate group of organic and inorganic phosphates.

TABLE III

Influence of Other Compounds

The concentration of the substrates was 4×10^{-3} *M* and that of the compounds tested was 1×10^{-2} *M*.

Compounds	Remained Activity			
	β-Glycero-phosphate	Phenyl-phosphate	Pyro-phosphate	Meta-phosphate
	%	%	%	%
$ZnCl_2$	51	68	73	49
$CaCl_2$	100	100	100	100
$MgSO_4$	100	100	100	100
$CuSO_4$	58	67	71	43
Na_2SO_4	100	100	100	100
Phloridzin	100	100	100	100
$MnCl_2$	83	75	80	70
NaF	11	10	16	60
Na_2MoO_4	2	5	16	3
CH_2ICOOH	93	90	90	90
Cysteine	105	110	105	110
KCN	100	115	105	100

TABLE IV

Effect of Heating on Km for Substrates

Substrates	Intact	37°, 24 hrs.	50°, 3 hrs.
Phenylphosphate	6.0×10^{-4} *M*	4.5×10^{-4} *M*	4.0×10^{-4} *M*
Pyrophosphate	3.1 " "	3.8 " "	8.3 " "
β-Glycerophosphate	20.0 " "	6.3 " "	5.3 " "
Metaphosphate	8.0 " "	20.0 " "	13.3 " "

SUMMARY

1. Sweet potato acid phosphatase was furher purfied with alcohol and $(NH_4)_2SO_4$, and the Q_p value for β-glycerophosphate of the final preparation was as high as 250,000. But specific phosphatases were not separated.

2. Starch chromatography of the purified preparation also failed

to separate specific enzymes.

3. Studies on the reactions with mixtures of two substrates lead to the conclusion that there is a non-specific phosphatase which acts upon phosphomonoesters, pyrophosphate, ATP, *etc.* The conclusion was supported also by the experiments in which the phenylphosphatase reaction was inhibited by the other substrates.

4. K_i's of arsenate and borate for the non-specific phosphatase were $4.9 \times 10^{-4}\,M$ and $8.3 \times 10^{-3}\,M$ respectively, and those for metaphosphatase activity were $1.6 \times 10^{-4}\,M$ and $3.0 \times 10^{-3}\,M$ respectively. Other compounds were tested for their influence upon the phosphatase reaction.

The authors wish to express their sincere gratitude to Prof. N. Takasugi for his kind advices in this work.

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THE CHEMICAL STRUCTURE OF THE TUBERCULIN ACTIVE PEPTIDE

I. ISOLATION OF β-ALANINE AS THE N-TERMINAL RESIDUE OF THE ACTIVE PEPTIDE

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One of the most interesting agent among the biologically active product of tubercle bacilli is the active principle of tuberculin. The protein nature of this agent appears to have been established by the extensive work of F. B. Seibert (*1*). However, the chemical structure of the active unit of this protein molecule is still undetermined.

Neverthless, in solving this problem, the presence of some well known evidence can not be overlooked; that is sometimes this active unit can exist in a state of relatively low molecular size, as described in the reports of various workers: by ultracentrifugation (Seibert and Svedberg), (*2*), and by ultrafiltration (Hoffman *et al.* (*3*) and Gough) (*4*). This fact has stimulated us to undertake the partial degradation of the tuberculin active protein into component peptides to observe the change of activity during the stepwise degradation, as well as learn the structure of an active peptide of the least possible length, if any of such is present.

Starting Material

The human tubercle bacilli, strain Kiyose No. 2 cultured 6 weeks at 38° in Lockemann's synthetic media, sterilized with steam and bacillary body were removed by paper filtration. From the filtrate thus obtained, two kinds of samples of active proteins were separated as follows.

a) *Phosphotungstic acid Precipitated Protein,* "*PWO-F*"—This was prepared according to the method of Sandor (*5*), with a slight modification: in order to remove the inactive protein which was precipitated by acidification with 10 *N*-sulphuric acid, added to the filtrate, concentrated in vacuo and the solution dialyzed. This was done before the addition of phospho-tungustic acid, instead of as in the case of direct treatment with this reagent.

b) *Tuberculin Protein Purified by Caolin Adsorption.* " β "—This was done according to the procedure of Maschmann and Küster's (*6*) original method, again with a modification: namely the use of cellophane for dialysis, instead of " Fisch-blase ", the obtained material designated " β " (Kasuya and Sato (*7*)).

Paper-Ionophoresis

As for the purification as well as the homogenicity test for the above proteins, paper zone electrophoresis was used according to the method of Kobayashi (*8*)), and Lockhart and Abraham (*9*). " Toyo " No. 50 filter papers (26×13 cm.) were used; samples were applied on every orginal line, as a streak, with a micropipett in an emulsion of 100 mg. per 1.0 ml. of phosphate buffer of pH 6.7. Ionophoresis was run for 16 hours, at 0,2 mA./cm., poetential gradient of 12 v/cm.

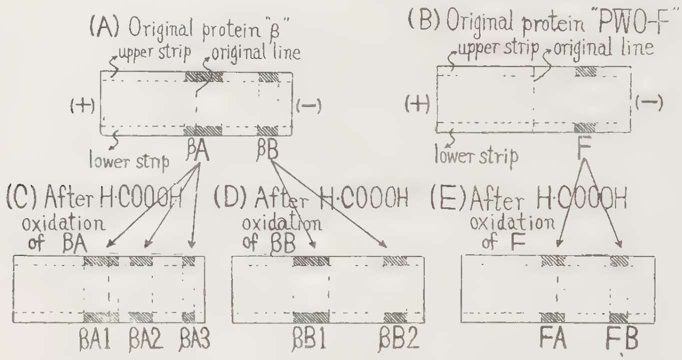

FIG. 1. Systematic separation of the peptides by paper-ionophoresis

After the run was finished, the papers were dried in a 100° oven. Three mm. wide of each pieces were cut off from both sides of the papers; and 0.2 per cent ninhydrin in water saturated butanol solution was sprayed on them, and then they were heated 5 minutes in a 100° oven. After the development of the color bands, (see Fig. 1 (A) and (B)) using these colored strips as a guide, the corresponding sites of the main paper were cut off, and eluted successfully in a small glass tubing with 10 per cent isobutanol. The eluted materials were lyophylized.

Performic Acid Degradation and Group Analysis

This operation was done as described by du Vigneaud *et al.* (*10*). One part of 80 per cent formic acid was mixed with one part of 30 per cent hydrogen peroxide, and the mixture left at room temperature for 25 minutes. About 5 mg. of the lyophylized material was treated with 2 ml. of the above performic acid solution at −10°

for 45 minutes, after which the majority of the performic acid was removed in vacuo as completely as possible. The group analysis of the resulted peptides was performed again by paper-ionophoresis as described above. Thr results are shown in Fig. 1, (C) to (E). " PWO-F " was separated in two bands, one acidic FA and the other basic FB. " βA " was separated into three bands, *i.e.* one acidic βA_1 and two basic βA_2 and βA_3; and the " βB " into βB_1 and βB_2. These bands were also eluted and taken to dryness similarly.

Isolation of the Single Peptide by Two-dimensional Paper-Chromatography

These peptides isolated by the paper-ionophoretic group analysis were still a mixture of some peptides, so these were separated from each other successfully by two-dimensional paper-chromatography, according to F. Sanger's method (*11*).

The solvent systems used were: 0.3 per cent NH_3-Phenol on one side, and Butanol-Acetic acid-water=4:1:5 on the other, according to the description of Sanger (11), the aqueous phase was separated and was poured into the bottom of a large glass cylinder, above which the main trough of the organic phase was settled. " Toyo " No. 50 filter paper of size 40×20 cm. was used. Each spot contained approximately 100 μg. of sample by the technique of repetition. After phenol front was reached at about 25 or 30 cm. run along the long axis of the paper, it was dried in an oven and then traces of phenol ware removed with steam as completely as possible. This paper was again rolled around the shorter axis and was dipped into butanol solution. After a solvent was run up to about 17 cm. or so, the paper was dried off from the solvent, 0,2 per cent ninhydrin was sprayed on both sides of the paper, and then it was heated to 70° for 5 minutes. The results are shown in Fig. 2.

As are seen in (b), (c) and (e), it is very interesting that βA2 βA3, and βB2 reveal a very peculiar spot βA2,2, βA3,2 and βB2,2 respectively. All these spots have very sharp, well defined deep ninhydrin color reactions with Rf. value of 0.33 in phenol, and 0.2 in butanol. In addition to that, as is evident in the spot βA2,2, in Table I, for example, all of these spots will show a very marked positive skin reaction for sensitized guinea pigs. These facts indicate that this spot may consist of a very simple peptide which is related to the essential part of the tuberculin activity.

Tuberculin Activity of the Peptide

The spots on the untreated papers were cut out with the aid of the ninhydrin developed paper (see Fig. 2), and were eluted as mentioned above. Skin tests were performed about each spot in comparison with the original iono-phoretically analysed groups of the peptides (Table I). For this experiment, several groups of guinea pigs, sensitized with paraffin emulsion of steam-killed human strain of tubercle bacilli, were used. Concentration of the peptide injected was estimated by the ninhydrin color reaction, and here, a scarcely discernible spot density was estimated as 2 to 3μg., and was injected as a 0.1 ml. physiological saline solution. The 2000$\times$ standard tubercu-

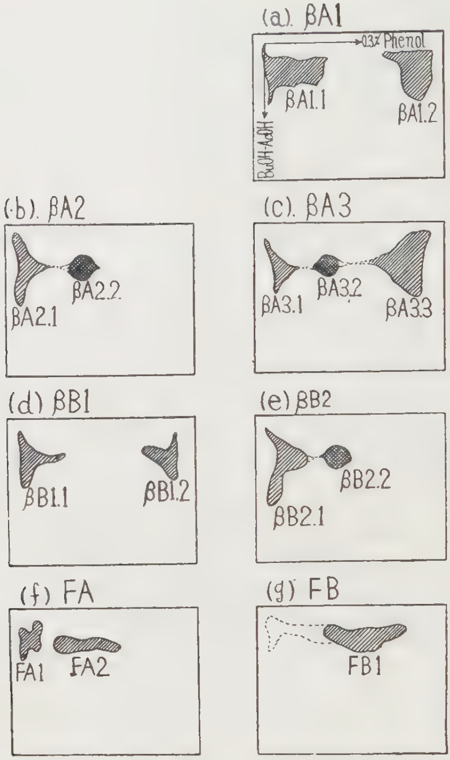

FIG. 2. Two dimensional paper-chromatogram of the paper-ionophoretically group-analysed peptides.

lin of The National Institute of Preventive Medicine was used as a control. Readings of the reactions were done twice after 24 and 48 hrs. from the inoculation. As is seen in Table I, βA2, βA3 and βB2 are very potent for this test, especially in βA2,2 which durates very strongly even after 48 hrs.

Amino Acid Composition of the Isolated Peptides

Single peptides isolated by two-dimensional paper-chromatography were taken to dryness. Each sample was sealed in a glass capillary with 2 or 3 drops of 6 *N*-HCl, and hydrolysed for 30 hours. at 110°, after which HCl were removed in vacuo as completly as possible. Then the resolution of amino acids was performed with two-dimensional paper-chromatography. The results are shown in Table II.

TABLE I

Result of the Skin Test of the Isolated Peptides

Peptide groups obtained by paper-iono-phoresis. (pH6.8)	Animals #201 24 hrs.	Animals #202 24 hrs.	Single peptids obtained by two-dimensional paper-chromato.	Animals #A 24 hrs.	Animals #A 48 hrs.	Animals #B 24 hrs.	Animals #B 48 hrs.
βA1	9×9* / 10×10	5×5 / 8×8	βA1.1	8×8 / 10×10	8×8 / 11×11	7×7 / 10×10	7×7 / 9×9
			βA1.2	3×3 / 6×6	4×4 / 7×7	0 / 0	0 / 0
βA2	11×11 / 17×17	7×7 / 10×10	βA2.1	9×9 / 11×11	8×8 / 11×11	6×6 / 9×9	9×9 / 7×7
			βA2.2	6×6 / 13×13	10×10 / 13×13	6×6 / 12×13	9×9 / 14×13
βA3	9×9 / 13×13	6×6 / 9×9					
βB1	4×4 / 7×7	9 / 3×3					
βB2	6×6 / 9×9	3×3 / 8×8					
FA.	—	—	FA.1	3×3 / 7×7	3×3 / 6×6	0 / 3×2	2×2 / 4×4
			FA.2	6×6 / 8×8	7×7 / 9×9	6×6 / 9×9	3×3 / 6×6
FB.	—	—	FB.1	5×5 / 9×9	4×4 / 6×6	3×3 / 6×6	3×3 / 6×6
			FB.2	9×9 / 13×14	9×9 / 14×14	9×9 / 12×12	11×11 / 13×13
O.T.**	10×10 / 15×15	9×9 / 13×13	O.T.	10×10 / 12×12	10×10 / 12×12	10×10 / 12×12	8×8 / 12×12

* Indicator designate diameter of swelling by m. m. (numerator) and of reddning (denominator).

** O. T. means 2000× standard old tuberculin.

DNP-Derivative of Active Peptide

The experiment was performed according to F. Sanger (*12*).

Experiment 1 :

" PWO-F ": To a solution of approximately 20 mg. of the material dissolved

TABLE II

Survey of the Peptide Isolated

No.	Name of peptide (*l.c.* fig. 2)	Strength in ninhydrin reaction	Strength of skin test	Amino acid compostion*
1	βA1.1	++	+++	[$CySO_3H$][Glu][Gly][X_1]**[Lys][Arg]
2	βA1.2	+	++	[$CySO_3H$][Glu]
3	βA2.1	++	+	[$CySO_3H$][Glu][Gly][Ala]
4	βA2.2	++	++++	[$CySO_3H$][Glu][Gly][Ala][X_1]
5	βA3.1	++	+++	[$CySO_3H$][Glu][Gly][X_1]
6	βA3.2	++	±	[Glu][Gly][X_2]***[X_1]
7	βB1.1	++	−	[Glu][Ser][Gly][Ala][X_2][Var]
8	βB1.2	++	−	[Glu][Ser][Gly][Ala][X_2][Var]
9	FA.1	++	±	[$CySO_3H$][Glu][Gly]
10	FA.2	++	+	[Gly][Thr][Lys][Arg]
11	FB.	+++	++++	[$CySO_3H$][Glu][Ser][Gly][Thr][X_2][Val][X_1]

* Due to the scaceness of the samples, the molecular weight determination and the quantitative estimation of the amino acid were not possible. So that the moral ratio of each amino acid is still unknown.

** X_1 was proved to be *N*-terminal residue, β-alanine by DNP-method.

*** X_2 is presumably α-amino-iso-butylic acid by Rf value in phenol chromatography.

in 1.0 ml. of water with 100 mg. of $NaHCO_3$ was added 100 mg. of DNFB dissolved in 2.0 ml. of ethanol at room temperature. This mixture was shaken vigorouly for 2 hours, then evaporated to dryness in vacuo. The excess of DNFB was extracted twice, each time with 3 ml. of ether. The residue was dissolved in 2.0 ml. of performic acid, and left for 45 minutes at $-10°$. Then the excess of performic acid was removed in vacuo, the residue was extracted three times, each time again with 3 ml. of ether (a), and then with 3 ml. of ethyl acetate (b) respectively. The fraction (a) and (b) thus extracted, containing DNP-peptides, were each concentrated to 0.2 m.l under reduced pressure, and were subjected to a column chromatography of silica gel. After elution of the bands with 50 per cent butanol-chloroform solution (B-C soln.), the solvent was removed in vacuo, and the residue was hydrolysed with a few drops of 11 *N* HCl, by boiling one min. over an open flame and the DNP-amino acid was extracted with ether (3×3 ml.). The residual pepetie was then hydrolysed completely with 6 *N* HCl at 105°, for 24 hrs., and the amino acids obtained were examined in two-dimensional paper-chromatography. Details are summarized in Table III.

TABLE III

Chromatography of DNP-Peptide on Silica Gel Column[12)]

Peptide	Source of DNP-peptide obtained	Solvent extracted	Solvent system: Stational phase	Solvent system: Moving phase	Rc value	Amino acid composition	N-Terminal residue
1	DNP-"PWO-F"→H·COOOH oxidized	ether (a)	water	50% B.C.	0.86	DNP-X_1 Glu.	β-Ala.
2	" "	Et. acetat (b)	"	"	0.78	DNP-X_1[Glu][Gly][$CySO_3H$]	β-Ala.
3	DNP-"β"→H·COOOH oxidixed	ether	IN. HCl	66% Me. Et.-Ketone	0.89	DNP-X_1[Glu][Gly][$CySO_3H$]	β-Ala.
4	"PWO-F"→Basic band (by paper-ionophoresis)→DNP→H·CO-OOH oxidized	purified on talc column (Sanger[13)])	"	"	0.85	DNP-X_1[Glu][Ser][Gly][$CySO_3H$][Ala][X_2][Val]	β-Ala.

Experiment 2 :

"β": 1.0 ml. of water contining 10 mg. of the substance and 50 mg. of $NaHCO_3$ was shaken with 2.0 ml. ethanol solution of 50 mg. DNFB for 2 hours. After drying in vacuo, the excess of DNFB was extracted twice, each time with 3 ml. of ether. The residue was treated with performic acid as mentioned above. After removal of the performic acid in vacuo, the residue was acidified with 3 drops of 6 *N* HCl and was extracted with ether. The deep yellow ether extract, after partial hydrolysis with a few drops of conc. HCl as mentioned before, gave DNP-amino acid, which was determined by paper-chromatography. (See the following section). The residual peptide was examined for its amino acid composition after complete hydrolysis with 6 *N* HCl, and on two-dimensional paper-chromatography. The details are written in Table III.

Paper-Chromatography of the DNP-N-Terminal Residue of the Active Peptide

DNP-amino acids as N-terminal residue of the active peptides were examined by paper-chromatography according to the solvent system of Biserte (*14*): toluene-pyridine-ethylenchlorohydrin-0.8 *N*-NH_3=5:1:3:3. Before spotting the samples, "Toyo" No. 50 filter paper of 40×2.5 cm. was dipped in the aqueous phase, and dried in air; and then while it was still damp the sample of DNP-amino acid was applied with a small micro pipette, in a spot scarcely exceeding 2 mm. according to the description of Li *et al.* (*15*) and Blackburn (*16*).

As was stated by these authors, the Rf values of DNP-amino acids are very unstable and change very easily with the slightest change in composition of the solvent; so that it is always neccessary to make a simultaneous run of the authentic samples. The relative Rf values of the DNP-N-terminal residue of the active peptide, together with some other reference DNP-amino acids, are illustrated in Fig. 3.

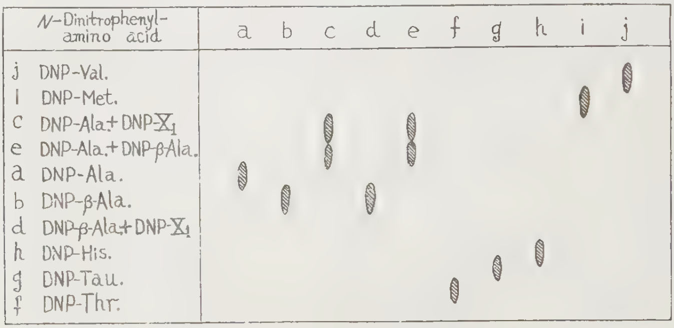

FIG. 3. Paper chromatogram of the DNP-amino acids.

As is seen in Fig. 3, the Rf of the DNP- amino acid in question (*i.e.* DNP-X_1) is higher than DNP-His. and DNP-Thr., but lower than DNP-Met. and DNP-Val. It is approximately the same as DNP-Ala. although always very slightly lower. This correlation is samewhat the same as in the case of the experiment with silica gel column using 3 per cent B-C. solvent. The mixed test (c) of DNP-X_1 and DNP-Ala. gave distinctly different results, although two spots closely approached to each other.

Identification of the N-Terminal Residue as β-Alanine

As a reference compound DNP-β-Ala. was prepared and mixed with DNP-X_1. No resolution of the spot was resulted (d), while the relation of DNP-Ala. to DNP-β-Ala. (e), in view of relative Rf, was quite the same as in the case of DNP-Ala. to DNP-X_1. (c). This evidence indicates that one part of the N-terminal residue of the active peptide is β-alanine.

As additional evidence in support of this view, the Rf value of one unknown ninhydrin positive spot among DNFB. untreated peptide hydrolysate (see Fig. 4) is very note worthy. Its Rf value was 0.67 in 0.3 per cent-NH_3-Phenol and 0.21 in BuOH-AcOH. This value is approximately the same as the value 0.66 (in phenol) described for β-Ala. (Block (*19*)).

DISCUSSION

On the result of trypsin digestion of the active protein has already

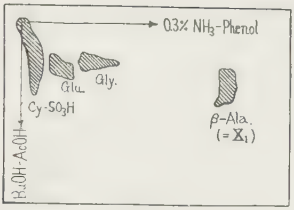

FIG. 4. Two dimensional paper-chromatogram of the shortest active unit peptide after hydrolysation.

been discussed by McCarter (*20*), and by Seibert (*21*); however, the observation of the change of activity in the course of partial degradation, including the structural study of the resulted peptide, has not yet appeared in the literature.

One method of partial hydrolysis is the conc. HCl-low temperature technique which was used by F. Sanger (*11*) in the study of the structure of crystalline insulin. However, for our purpose, to obtain the shortest possible peptide having the same biological activity, the performic acid degradation at low temperature was more preferable.

The presence of cystine as a component amino acid of the tuberculin active protein has been reported by various workers (Seibert (*17*); Jones *et al.* (*18*)) even though present in only a relatively small amount. It is obvious, therefore, that the -S-S- bridge of cystine must have been affected by performic acid oxidation to yield cysteic acid containing peptide, as has been described in this paper. In addition, another attack point of performic acid in the peptide chain is, as was known, tryptophane. Its color reaction of Adamkiewiz is stated essential for the biological activity of tuberculin protein (*3*), however, in our experience, the destruction of the tryptophane seems to have no appreciable correlation with biological activity in term of skin reaction.

The fact that a structural study of tuberculin protein has not been made, is probably due to the difficulty of obtaining crystalline protein in uniform homogeneity as in the case of insulin. Neverthless, for the purpose of searching out a definite peptide chain as the active unit, the method of purification with a combination of the group analysis by paper-ionophoresis, and two dimensional paper-chromatography, as was used here, provide a satisfactory result.

By the DNP method, applied before or after a performic acid oxidation, followed by silica gel column chromatography, or by paper-ionophoresis and two dimensional paper-chromatography, we have obtained two kinds of active peptide with different lengths; *viz.*,

one basic: β-Ala. [Cy.-SO_3H] [Glu.] [Gly.] [Ser.] [Ala.]-

[Val.] [X_2] .... (I)*
one neutral β-Ala. [Cy.-SO_3H] [Glu.] [Gly.] .... (II)
and one inactive peptide : [Gly.] [Thr.] [Lys.] [Arg.]...... (III)

Here (II) was found to be an active peptide of least possible length, which seemed to be contained also in (I), and may be recognized as a unit of active peptide. This peptide was that one which was found in βA22 and βA31 as a well-defined, concentrated spot (Fig. 2).

As clues in defermining the N-terminal residue (DNP-X_1), we had the following data available: (i) DNP-peptide obtained from DNP-" PWO-F " yielded peptide [CySO_3H] [Glu.] [Gly.] after the removal of DNP-X_1 ; (ii) DNP-" β " yielded again [CySO_3H] [Glu.] [Gly.] [DNP-X_1], and the DNP-X_1 was proved to be DNP-β-Ala. by paper chromatography with an appropriate solvent system ; and (iii) the Rf value of DNBF untreated unidentified ninhydrin positive spot on the paper chromatogram of hydrolysed βA2.2 or βA3.2 (Fig. 4) was in good agreement with the literature value of β-alanine. Basing judgement upon all this evidence, it is permissible to say that the N-terminal residue of the active peptide is β-alanine.

In as much as the occurrence of β-alanine is uncommon in nature, except in pantothenic acid or in carnosin and anserin, the presence of this rare amino acid as N-terminal residue of the biological active unit of tuberculin protein is very interesting. The physiological activity of β-alanine or its rerated peptide will be described elsewhere.

A report concerning another N-terminal amino acid residue and some sequence of them will be report in the near future.

SUMMARY

1. A basic tuberculin active protein has been isolated from heat-killed culture filtrate of tubercle bacilli (human strain), either by phosphotungstic acid precipitation or by caolin adsorption, the uniformity of which was ascertained by paper-electro-ionophoresis at pH 6.8 phosphate buffer.

2. Through performic acid degradation, this basic active protein

* In another occasion, the counter-current distribution performed with the performic acid oxidized DNP-derivative of a tuberculin protein (PPD-S (*21*), between the system of *n*-BuOH and 5 per cent-NH_4OH (*22*), yielded DNP-β-alanine peptide and DNP-glutamic acid peptied; the former was agreed to (I) and the later to the combination of (II) and (III), in regard to their amino acid composion.

is devided into two cysteic acid-containing peptides: *viz.* one acidic and one basic indicating that these two peptides were linked together with at least one -S-S- bridge.

3. Among the three peptides, only the basic and neutral one are tuberculin active, concerning which a detailed study has been made in this paper; while the acidic one, which is inactive, will be discussed in a subsequent paper.

4. The shortest active unit of these peptides, *viz.* the neutral one, seems to be derived from the basic one, is composed of β-alanine, glutamic acid, glycine, and cysteic acid. The N-terminal residue of this peptide identified as β-alanine by paperchromatography of DNP-derivative.

In conclusion a grateful acknowledgement is made for the financial aid of the Ministry of Education and of the Ministry of Welfare. The authors also offer their sincers thanks to Prof. C. Nishino the director of the 2nd. Tokyo National Hospital, for his valuable advice; and also to Mr. J. Goto for his assistance throughout in the chemical analyses and in the animal experiments.

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THE PRESENCE OF XANTHURENIC ACID IN THE FRUIT-FLY, DROSOPHILA MELANOGASTER

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When tryptophan metabolites of the fruit-fly, *Drosophila melanogaster*, are examined by means of paper chromatography, two diazo-positive substances are found (*1*). One of them was proved to be 3-hydroxykynurenine, but the other has not yet been fully identified, though it might be xanthurenic acid or its derivatives, judging from its Rf value and various color reactions (*1*). Since this work, attempts have been made to compare the unidentified substance with synthetic xanthurenic acid in various points. The present paper gives the results of the comparison of the two substances in Rf value by paper chromatography, paper electrophoretic behaviors, color reactions, and ultraviolet absorption. The paper also deals briefly with the presence of diazo-positive substances in the eye-clore mutant strains of *Drosophila.*

MATERIALS

The flies studied in this work were a wild type of *D. melanogaster* and the following eye-color mutant strains: the vermilion (*v*), cinnabar (*cn*), scarlet (*st*), brown (*bw*), sepia (*se*), vermilion brown (*v bw*), cinnabar brown (*cn bw*), brown scarlet (*bw st*), vermilion sepia (*v se*), cinnabar sepia (*cn se*), and sepia scarlet (*se st*) strains, the latter six being double recessive strains. As to the eye-colors of the double recessive strains, those of the *v bw*, *cn bw*, and *bw st* strains are white. Those of the *v se*, *cn se*, and *se st* strains are yellow at first on emergence and then (after one day) turn into the same black brown as that of *se*.

The medium for the growing of the flies had the following composition: agar, 12.5 g.; sugar, 50 g.; malted rice, 125 g.; K_2HPO_2, 0.90 g.; water, 900 ml. After cooking and bottling, the medium was seeded with the yeast. All cultures were kept at about 25°C.

METHODS

Extraction and Paper Chromatography—Three hundred flies, male and female, were anaesthetized with ether and decapitated. The heads were homogenized with 1 ml.

of 80 per cent ethanol and centrifuged. Then 0.15 ml. of the supernatant fluid was applied little by little to a single spot of reference mark of a strip of filter paper (Toyo No. 50, 1.5×40 cm.) and subjected to chromatography. In the cases of the thorax and abdomen, males and females were separated. The posterior parts of one hundred flies (male or female) were gathered, homogenized with 1 ml. of 80 per cent ethanol, and treated in the same way as the heads. One-demensional chromatograms were developed with either of the following solvents:

(i) B.A.W.—*n*-butanol:acetic acid:water (4:1:5)

(ii) M.B.B.W.—methanol:benzene:*n*-butanol:water (2:1:1:1) (*2*)

Color reactions—After being developed and dried, the chromatogram was inspected under ultraviolet rays and the areas of fluorescent spots were marked with a pencil. Then the following color tests were made on the paper:

(i) Ehrlich's diazo reaction—The paper was sprayed with Ehrlich's diazo reagent and then with 14 per cent ammonia water. The diazo reagent was a mixture (20:1) of the two solutions: (a) 5 g. sulfanilic acid dissolved in 50 ml. conc. HCl and made up to 1 *l.* with distd. water; (b) 0.5 per cent aqueous solution of $NaNO_2$.

(ii) Millon's reaction (*3*)

(iii) Ammoniacal silver nitrate reaction (*4*)

(iv) Ferric chloride reaction (*3*)

(v) Ninhydrin reaction

(vi) Pauli's reaction

(vii) Ehrlich's aldehyde reaction (*5*)

(viii) Dragendorff's reaction (*6*)

(ix) Tsuda's reagent (*7*)—The paper was sprayed with 0.2 per cent $NaNO_2$ in 0.1 N HCl, dried at 50° for a short time, and then sprayed with 0.2 per cent β-diethylaminoethyl-α-naphthylamine oxalate in ethanol.

(x) Potassium permanganate reaction (*4*)

Paper Electrophoresis—The ethanol extract of the heads of three hundred flies was placed along the starting line 3 cm. from the margin of the filter paper sheet (24×25 cm). After developing with the solvent B.A.W.one-dimensionally, area of the paper corresponding to the unidentified diazo-positive substance was cut out and eluted with 80 per cent ethanol. The eluate was then applied to the middle of a filter paper strip (1.5×40 cm), and submitted to electrophoresis under the following conditions: Sϕrensen's 1/15 M phosphate buffer (pH 5.3–8.0); approximately 400 volts, and 3.8–7.0 mA. After running for about 6 hrs., the paper was removed, dried, and visualized by Ehrlich's diazo reaction.

Ultraviolet Absorption—The ethanol extract of the heads of three thousand flies, male and female, was applied to three sheets of paper (24×25 cm) along the starting line 3 cm. from the margin of a sheet. After developing with the solvent B.A.W. one-dimensionally, area of the paper corresponding to the unidentified diazo-positive substance was cut out and eluted with 1/15 M phosphate buffer (pH 7.4). The ultraviolet absorption of this eluate was measured between 230 and 370 mμ using

a Beckman spectrophotometer, model DU.

Paper chromatography, paper electrophoresis, various color tests, and ultraviolet absorption measurement were also made on synthetic xanthurenic acid for the purpose of comparison.

RESULTS

Rf Values—When chromatographed and examined by Ehrlich's diazo reaction, the ethanol extract of the head of wild-type flies gave two positive spots as shown in Fig. 1, where spot 2 corresponds to 3-hydroxykynurenine, and spot 1 to the unidentified substance. The results obtained on the Rf values of synthetic xanthurenic acid and the diazo-positive substances of the fly are given in Table I, where it will be seen that spot 1 showed the same Rf value as that of xanthurenic acid for either of the two kinds of solvents used.

TABLE I

Rf values of Synthetic Xanthurenic Acid and Diazo-positive Substances of the Fly

Spot	Solvent	
	B.A.W.	M.B.B.W.
Spot 1	0.50	0.65
Spot 2	0.38	0.32
Synth. xanthurenic acid	0.50	0.65

FIG. 1. Paper chromatogram of diazo-positive substances in the wild type of *D. melanogaster*. Solvent: B.A.W.

Color Tests—As shown in Table II, the results of various color tests on synthetic xanthurenic acid were quite parallel to those on spot 1 of the fly. Fluorescence was very weak in both.

Paper Electrophoresis—The wild and *bw* strains were examined. Both synthetic xanthurenic acid and spot 1 of the fly migrated to the anode with the same velocity. On the contrary, 3-hydroxykynurenine (spot 2) migrated to the cathode as already reported (*5*), and spots 1 and 2 were thus easily separable.

Ultraviolet Absorption—The *bw* and *st* strains were examined. Two peaks (at 242 mμ and 336 mμ) were found in both synthetic xanthurenic

TABLE II

Color Reactions of Spot 1 of the Fly and Synthetic Xanthurenic Acid

Reaction	Spot	
	Spot 1	Synth. xanthurenic acid
Ehrlich's diazo	+(rose red)	+ (rose red)
Million	+(orange)	+ (orange)
Pauli	+	+
Ammonical silver nitrate	+(weak sensitivity)	+ (weak sensitivity)
$FeCl_3$	+(dark green. weak sensitivity)	+ (dark green. weak sensitivity)
$KMnO_4$	+(very weak sensitivity)	+ (very weak sensitivity)
Ninhydrin	−	−
Ehrlich's aldehyde	−	−
Dragendorff	−	−
Tsuda	−	−

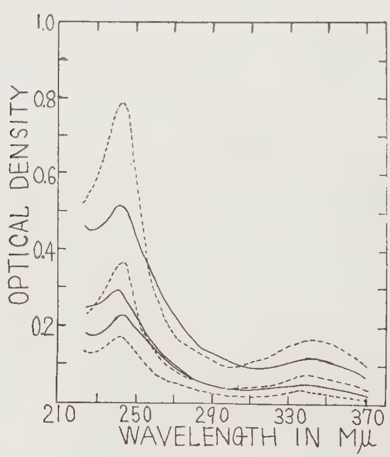

FIG. 2. Ultraviolet absorption curves of synthetic xanthurenic acid (- - - - - -) and the unidentified diazo-positive substance of the fly (———).

acid and spot 1 of the fly, as illustrated in Fig. 2.

Presence of the Diazo-positive Substances in the Eye-color Mutant Strains—The presence of the unidentified diazo-positive substance (spot 1) and 3-hydroxykynurenine (spot 2) in various eye-color mutant strains was examined by paper chromatography. At the same time, kynurenine was examined by its fluorescence on the chromatogram. The results of these examinations are given in Table III.

TABLE III

Presence of Tryptophan Metablites in the Eye-color Mutants of the Fly

Type	Substance		
	Kyurenine	3-OH-kynurenine	Xanthurenic acid (spot 1)
Wild	+	+	+
v	−	−	−
cn	+	−	−
st	+	−	+
bw	+	+	+
se	+	+	+
v bw	−	−	−
cn bw	+	−	−
bw st	?	−	−
v se	−	−	−
cn se	+	−	−
se st	?	−	+

Other Observations—In the strains thus far studied, the substance represented by spot 1 was more abundant in the head than in the posterior part, and no sexual differences were recognized concerning the spot 1.

The pupa of the wild strain was also studied. The substance of spot 1 was much more abundant in the imago than in the pupa at any period.

DISCUSSION

From the striking parallelism between the "unidentified" diazo-positive substance of spot 1 in the fly and synthetic xanthurenic acid shown in their Rf values, color reactions, paper electrophoretic behaviors, and

ultraviolet absorption curves, the unidentified substance may presumably be identical with xanthurenic acid. This is very interesting, for in mammals xanthurenic acid has been postulated to be an abnormal metabolite of tryptophan (*8, 9, 10*).

As xanthurenic acid in mammals is known to be formed from kynurenine by way of 3-hydroxykynurenine (*10*), it would be interesting to ascertain whether these metabolites is present in the eye-color mutant strains of the fly. Indeed, in the wild and the *bw* and *se* strains, which exhibited spot 1 (xanthurenic acid) on paper chromatograms, kynurenine and 3-hydroxykynurenine were detected (Table III). On the other hand, it is to be noted that xanthurenic acid was absent in the *v* and *cn* strains, for tryptophan metabolism is known to be interrupted on its way from tryptophan to kynurenine in the *v* strain and from kynurenine to 3-hydroxykynurenine in the *cn* strain (*11*). It can well be understood also that the *v bw*, *cn bw*, *v se*, and *cn se* strains were negative for xanthurenic acid, for both the *v* and the *cn* strain were negative to it. The *st* strain gives an interesting instance. This strain was positive for xanthurenic acid (spot 1), but negative for 3-hydroxykynurenine (spot 2) (Spot 1 was strongly positive in the head but negative in the posterior part; spot 2 was negative in both the head and the posterior part.) As this is based only on paper-chromatographic determinations by the diazo reaction, it cannot be concluded that 3-hydroxykynurenine was absolutely absent in the *st* strain. The *bw st* strain also gave an interesting instance in regard to xanthurenic acid. Though both the *bw* and *st* strain were positive for xanthurenic acid, the *bw st* strain was negative. No satisfactory explanation has yet been found for this. On the other hand, the *se st* strain was positive for xanthurenic acid though less in extent than the *se* and *st* strains. These results on the *st*, *bw st*, and *se st* strains present some interesting problems in regard to the tryptophan metabolism in the fly, the nature of the *st* strain, the double recessive white eye, and the relation between tryptophan metabolism and pterin metabolism.

It is noteworthy that in all the strains which gave spot 1 xanthurenic acid was more abundant in the head than in the posterior part. This fact suggests that a quinoline ring formation may be much more active in the head than in the posterior part. It has lately been reported that xanthommatin (a kind of ommochrome, [purified from the mucous secretion of a butterfly, *Vanessa urticae*) is a condensate of two molecules of 3-hydroxykynurenine (*12*), and also that one of the two molecules forms a quinoline ring in the same way as in the formation of xanthurenic

acid from 3-hydroxykynurenine. Therefore, the fact that xanthurenic acid was abundant in the head of the fly suggests that some eye pigments of *Drosophila* may involve quinoline ring formation somewhere in their process of formation.

SUMMARY

1. An unidentified diazo-positive substance of the fruit-fly, *D. melanogaster*, was compared with synthetic xanthurenic acid in regard to the Rf value in paper chromatography, color reactions, paper electrophoretic behaviors, and ultraviolet absorption. It was found that both substances were identical.

2. The wild strain and various eye-color mutant strains (including five recessive and six double recessive strains) were examined for the presence of xanthurenic acid. The wild, *st*, *bw*, *se*, and *se st* strains were positive for it, while the *v*, *cn*, *v bw*, *cn*, *bw*, *bw st*, *v se*, and *cn se* strains were negative. These results are discussed from the point of view of tryptophan metabolism.

The authors wish to express their gratitude to Professor Yahito Kotake of the Wakayama Medical College for the generous gift of synthetic xanthurenic acid, and to the Mizuno Laboratory, Faculty of Pharmacy, Kanazawa University, for the measurements of ultraviolet absorption. Thanks are also due to Dr. G. Tomita of the Tôhoku University for his kindness in reviewing this manuscript.

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CELLULOSE-SPLITTING ENZYMES

V. PURIFICATION OF IRPEX CELLULASE AND ITS ACTION UPON *p*-NITROPHENYL β-CELLOBIOSIDE

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In a previous paper Nisizawa and Kobayashi (*1*) reported that the reducing sugars formed by the action upon hydrocellulose of a cellobiase free preparation of *Irpex* cellulase* were composed mainly of cellobiose together with a very small quantity of glucose and that as a result of the cellulase action the copper value of hydrocellulose recovered from the reaction mixture was found to increase almost proportionally to the decrease in weight of the substrate. Based on these findings it was suggested that *Irpex* cellulase might split cellobiose unit successively from the end of the cellulose chain. In this respect the mode of action of *Irpex* cellulase seems to resemble that of β-amylase which likewise cuts off maltose unit from the end of the amylose molecule (*3*).

Subsequently it was found that *p*-nitrophenyl β-cellobioside, a heterobioside corresponding in structure to cellotriose in that it is composed of three units, was split by a cellobiase free preparation of *Irpex* cellulase. A further finding was that the resulting reducing power of the incubation mixture was significantly larger than the value calculated from the amount of the liberated *p*-nitrophenol on the assumption that the reducing sugar produced was consisted solely of cellobiose, whereas the reducing power was insufficient to account for that of the incubation mixture if the substrate had been split into one molecule of *p*-nitrophenol and two of glucose (*4*). On the other hand, when a cellobiase preparation of *Irpex* which had been freed from cellulase was used, the molar ratio of glucose to *p*-nitrophenol as cleavage products was close to 2 :1, assuming that no sugar other than glucose were formed. From these results, it may be considered probable that *Irpex* cellulase splits *p*-nitro-

* It has been ascertained that the cellobiase of *Irpex lacteus* is identical with the ordinary β-glucosidase which hydrolyses aryl β-glucosides (2).

phenyl β-cellobioside partly into aglucone and cellobiose and partly into glucose and p-nitrophenyl β-glucoside, whereas *Irpex* cellobiase hydrolyzed the β-cellobioside completely into its constituents, *i.e.* two molecules of glucose and one molecule of aglucone. Such a behavior of *Irpex* cellulase toward p-nitrophenyl β-cellobioside that this enzyme cuts off from β-cellobioside two units at a time might be taken to support the above mentioned view that the action of this cellulase primarily in the cleavage of two glucose units from the end of cellulose molecule.

On the other hand, it was reported by Helferich and Petersen (*5*) that the α-maltosides of phenol and other aglucones were never split by malt amylase. Afterwards, Myrbäck and Nycander (*6*) found that maltotriose was not hydrolyzed by β-amylase, and more recently Akabori and his collaborators (*7*) indicated the resistance of phenyl α-maltoside toward crystalline Taka-amylase. All these findings led us to the suspicion that the hydrolysis of p-nitrophenyl β-cellobioside by our cellulase preparation might have been due to the presence of a specific β-cellobiosidase contaminated in the preparation. Accordingly an extensive purification of *Irpex* cellulase was carried out in order to decide the problem of the identity of cellulase and β-cellobiosidase.

EXPERIMENTAL AND RESULTS

I. Substrates

(a) p-Nitrophenyl β-cellobioside, prepared according to Nisizawa and Wakabayashi (*8*), m.p. 243–245°, $[\alpha]_D^{10} = -84.7°$ (in 40 per cent methanol).
(b) Carboxymethyl cellulose (CMC), sodium salt, degree of etherification 0.427.

II. Enzyme Assay

The cellulase activity of each enzyme preparation was estimated with CMC and p-nitrophenyl β-cellobioside as substrate. Also the activity of cellobiase, β-glucosidase (salicin as substrate) and amylase was measured in most cases. p-Nitrophenol liberated was determined colorimetrically and reducing sugar was by the Shaffer-Hartmann-Somogyi's method.

III. Purification of Cellulase

The submerged culture of *Irpex lacteus* was carried out at 25–27° in a medium of the following composition which had been found to be the most suitable for the purification of cellulase (*9*).

Filter paper	2.6 g.	K_2HPO_4	0.25 g.
Lactic acid	0.13 ,,	$MgSO_4 \cdot 7H_2O$	0.06 ,,
NH_4NO_3	0.5 ,,	Water	150 ml.

After incubation for about 15 days, the culture fluid was filtered and 5.5 l. thereof served as starting material for the purification of cellulase, the procedure of which was summarized in Fig. 1. The specific activity of each cellulase preparation is given in Table I together with activities toward other substrates.

Fig. 1. Precedure of the purification of *Irpex* cellulase.

Starting solution (5.5 l.)
| Concentrate, dialyze for 4 days
150 ml.
(*Enz. soln. 1*)
| Add acetone (68%), centrifuge

Supernatant

Precipitate
| Dissolve in 100 ml. water, centrifuge

Suppernatant
(*Enz. soln. 2*)
| Add acetone (50%), centrifuge

Insoluble residue

Precipitate
| Dissolve in 100 ml. water
(*Enz. soln. 2′*)

Supernatant
| Add acetone (63%), centrifuge

Precipitate
| Dissolve in 100 ml. water, centrifuge

Supernatant

Insoluble residue

Supernatant
(*Enz. soln. 3*)
| Add acetone (60%), centrifuge

Precipitate
| Dissolve in 50 ml. water, centrifuge

Supernatant

Insoluble residue

Supernatant
(*Enz. soln. 4*)
| Add acetone (58%), centrifuge

Precipitate
| Dissolve in 50 ml. water, centrifuge

Supernatant

Insoluble residue
(very small amount)

Supernatant
(*Enz. soln. 5*)
| Add acetone (61%), centrifuge

Precipitate
(somewhat crystalline)
| Dissolve in 30 ml. water*
(*Enz. soln. 6*)
| Add acetone (70%), centrifuge

Supernatant

Precipitate
(crystals)
| Dissolve in 20 ml. water*
(*Enz. soln. 7*)
| Add acetone (75%), centrifuge

Supernatant

Precipitate
| Dissolve in 10 ml. water*
(*Enz. soln. 8*)

Supernatant

* No insoluble matter remained.

TABLE I

Activities of Enzyme Fractions toward Various Substrates

Enzyme fraction[1]	Total volume ml.	Total protein-N mg.	Activity toward: CMC (0.294%) Cleavage %	CMC (0.294%) k×Total volume[2]	CMC (0.294%) Specific activity[3]	Cellobiose (0.116%) ml.[4]	Salicin (0.33%) ml.[4]	Starch (0.25%)[4]	*p*-Nitrophenyl β-cellobioside (0.157%) ml.
1	150	14.40	32.3	1780	124	0.60	0.90	9.70	—
2	100	6.70	41.4	1522	227	0.40	—	8.30	—
2′	100	—	—	—	—	0.30	—	0.40	—
3	100	4.05	28.6	1051	260	0.0	—	5.10	—
4	50	1.90	30.6	562	295	0.0	—	2.30	—
5	50	1.564	28.6	526	336	—	—	0.50	—
6	30	0.683	21.5	233	341	—	—	0.0	—
7	20	0.380	17.7	130	342	—	0.0	0.0	—
8	10	0.183	17.0	63	342	—	—	—	0.029[5] (6) (f=0.00253)

(1) Numbers of enzyme fractions are the same as in Fig. 1. (2) k was calculated from the equation $x = kt^n$, where x represents the cleavage per cent after reaction time of 150 minutes with CMC as substrate and n was chosen as 1/5 based on unpublished observation. (3) k/mg. protein-N. (4) ml. of 0.005 *N* thiosulfate consumed per ml. of reaction mixture. (5) mg. *p*-nitrophenol liberated per ml. of reaction mixture. (6) $f = k' \times$ total volume/mg. total protein-N, where k′ represents the velocity constant of the first order reaction. All enzymic reactions were carried out at 30° in 0.5 *M* acetate buffer of pH 4.0.

As can be seen in Table I, about three-fold purification of *Irpex* cellulase as compared with the starting culture solution has been achieved whereby a crystalline preparation has been obtained. The specific activity of the purified preparation remained unchanged by further recrystallization from dilute acetone. The crystals (Fig. 2) were almost uniform in shape and no change in crystal form was observed upon repeated recrystallization.

It would appear, therefore, that the obtained crystals might be regarded as pure cellulase in spite of their relatively low activity. The final three preparations (*Enzyme soln.* Nos. 6–8) are completely free of cellobiase, β-glucosidase and amylase while, the activity toward *p*-nitrophenyl β-cellobioside is still retained. These results may be taken as an indication that *Irpex* cellulase itself is responsible for the hydrolysis of *p*-nitrophenyl β-cellobioside.

IX. Action of Irpex Cellulase upon p-Nitrophenyl β-cellobioside

As indicated in the above experiment the β-glucoside bond between aglucone and

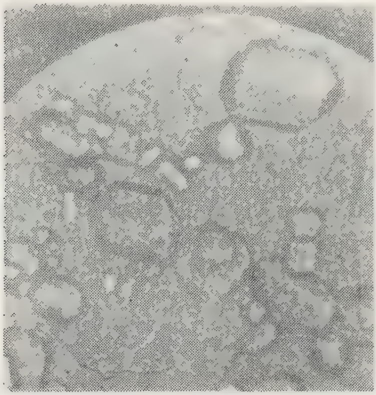

FIG. 2. Crystals of highly purified cellulase preparation from *Irpex lacteus*. ×600.

cellobiose residue of *p*-nitrophenyl β-cellobioside was split by *Irpex* cellulase (Table I). The time course of the hydrolysis of this bond by purified cellulase preparation was followed and the results were presented in Fig. 3 (Curve I). The degree of hydrolysis was calculated from the amount of liberated aglucone. However, when calculated on

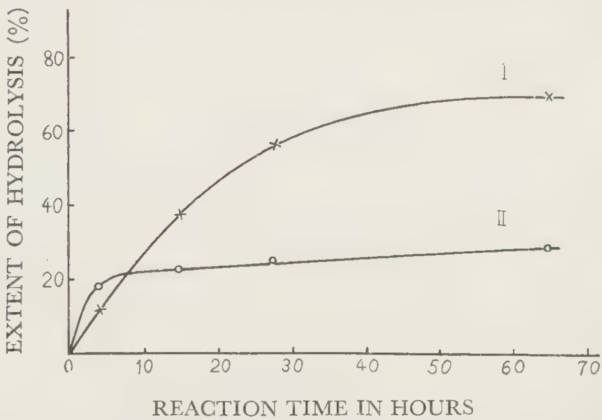

FIG. 3. Hydrolysis of *p*-nitrophenyl β-cellobioside by *Irpex* cellulase. Curve I : cleavage of agluconic bond. Curve II : cleavage of holosidic bond. Substrate concentration, 0.0034 *M*.

the basis of reducing power of the incubation mixture assuming that the sugar liberated was cellobiose alone, higher values of the degree of hydrolysis were obtained, *i.e.* 10–20

per cent higher than those calculated from liberated aglucone. These results were quite similar to those described in a previous paper (*4*).

Since the cellulase preparation used in this experiment was proved to be without action upon cellobiose, as shown in Table I, it appeared hardly likely that cellobiose was hydrolyzed after it had been released from the substrate by the action of cellulase. Consequently it may be considered probable that the terminal glucose residue of the substrate molecule must also be split off by the action of cellulase, thus contributing to the reducing value of the reaction mixture. The amount of glucose thus formed may be calculated from the reducing value of the incubation mixture and the amount of cellobiose which is equivalent to liberated aglucone. The results are presented in Fig. 3 (Curve II).

The figure clearly indicates the difference in the progress of the hydrolysis of agluconic bond. The course of the cleavage of the agluconic bond follows nearly the first order reaction with a velocity constant of 0.013 (*t* in *hr.*), whereas the reaction rate of the holosidic bond hydrolysis is relatively fast in the early stage but soon drops till the hydrolysis almost stops at about 30 per cent cleavage.

V. Inhibition of the Hydrolysis of p-Nitrophenyl β-cellobioside by Split Products

As the anomalous time course in the enzymic cleavage of the holosidic bond of *p*-nitrophenyl β-cellobioside suggested that the cleavage might be inhibited by split products, the effect of the cleavage products and that of some other related compounds upon the enzyme action were examined. The results are shown in Tables II–VII.

TABLE II

Inhibition by Phenol

Concentration (*M*) of		Hydrolysis (%) after 300 *min.*	
Substrate	Phenol	Agluconic bond	Holosidic bond
0.0034	0	13.5	32.4
	0.0026	13.5	32.4
	0.2142	6.7	32.1

From these tables, it may be pointed out that the hydrolysis of the agluconic bond is hardly affected by added glucose, cellobiose and phenyl β-glucoside even in concentrations 63 times as much as that of the substrate, whereas the rate of the hydrolysis of the holosidic bond is reduced to 1/2–1/3 of the control by the presence of these substances in smaller concentration than that of the substrate (Tables III, IV and V). On the contrary the inhibitiory action of phenol is quite different from that of the sacharides mentioned above ; namely, the hydrolysis of the holosidic bond is not inhibited by phenol of the high concentration of 70 times as much as that of the substrate, but the cleavage

TABLE III

Inhibition by Glucose

Concentration (M) of		Hydrolysis (%) after 320 minutes	
Substrate	Glucose	Agluconic bond	Holosidic bond
0.0034	0	16.0	19.2
	0.00065	16·0	12.0
	0.0013	15.2	8.6
0.00057	0.0026	14.4	6.0
	0.0036	14.0	—

TABLE IV

Inhibition by Cellobiose

Concentration (M) of		Hydrolysis (%) after 230 minutes	
Substrate	Cellobiose	Agluconic bond	Holosidic bond
0.0034	0	13.4	35.0
	0.00065	13.4	27.0
	0.0013	13.4	22.7
	0.0026	13.4	17.0
0.00057	0.036	11.0	—

TABLE V

Inhibition by Phenyl β-Glucoside

Concentration (M) of		Hydrolysis (%) after 300 *min.*	
Substrate	Glucoside	Agluconic bond	Holosidic bond
0.0034	0	11.1	18.3
	0.0013	9.6	11.8
	0.0026	10.6	8.7

rate of the agluconic bond is depressed to nearly a half of the control by phenol of the same concentration. Further it was found that gluconate as well as β-galactoside, both of which are related in structure to split products showed no inhibition upon the hydrolysis of both holosidic and aluconic bonds (Tables VI and VII).

From these results it may be considered as certain that the marked reduction in the rate of the hydrolysis of the holosidic bond, as illustrated in Fig. 3, can be attributed, at least in part, to the inhibition by the split products such as *p*-nitrophenyl β-glucoside, cellobiose or glucose, but not *p*-nitrophenol.

TABLE VI

Inhibition by Ca Gluconate

Concentration (M) of		Hydrolysis (%) after 240 minutes	
Substrate	Gluconate*	Agluconic bond	Holosidic bond
0.00057	0 0.0034	32.2 23.1	57.5 55.5

* As gluconate has been dissolved in 0.05 M acetate buffer of pH 4.0, a part of it might be reversibly converted into its lactone.

TABLE VII

Inhibition by Phenyl β-Galactoside

Concentration (M) of		Hydrolysis (%) after 300 minutes	
Substrate	Galactoside	Agluconic bond	Holosidic bond
0.0034	0 0.0013	11.1 10.1	18.3 18.5

Therefore the dissociation constants (K_i) of cellulase-inhibitor compounds was calculated from the data in Tables III–V according to the equation, $I = \frac{[S]}{K_i + [S]}$, where I and [S] represent inhibition degree and inhibitor concentration respectively. As shown in Table VIII the values of K_i were found to be in an order of 10^{-3}, whereas the

TABLE VIII

Dissociation Constants of Cellulase-Inhibitor Compounds

Inhibitor	Dissociation constant
	mole/lit.
Glucose	0.001
Cellobiose	0.0025
Phenyl β-glucoside	0.0024

K_m value of cellulase—p-nitrophenyl β-cellobioside compound for the hydrolysis of agluconic bond was 0.04, as obtained from the activity-pS-curves (Fig. 4). Hence it would be possible that the split products might inhibit also the hydrolysis of the agluconic bond. Actually this was not the case. These findings might suggest the presence on the cellulase molecule of two distinct active centers, the one responsible for the hydrolysis

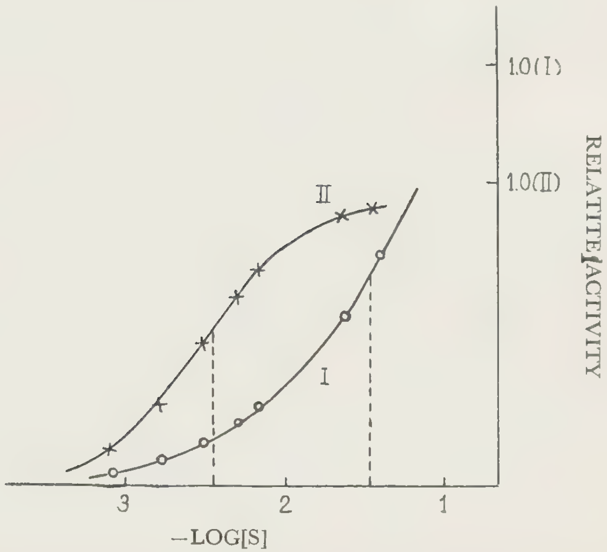

FIG. 4. Activity-*p*S-curves of *Irpex* cellulase for the hydrolysis of *p*-nitrophenyl β-cellobioside. Curve I : cleavage of agluconic bond (K_m, 0.04). Curve II : cleavage of holosidic bond (K_m, 0.004).

of the agluconic bond and the other for that of the holosidic bond of *p*-nitrophenyl β-cellobioside, the latter center exhibiting high affinity for split products.

DISCUSSION

From the culture fluid of a wood rotting fungus, *Irpex lacteus*, a crystalline substance was isolated which exhibited a relatively high activity of cellulase with CMC as substrate. This crystalline preparation is practically free of cellobiase, aryl β-glucosidase (salicin as substrate) and amylase, all of which were present in the starting crude enzyme solution. The specific activity of this preparation did not change upon repreated recrystalization. These findings might be taken to indicate that this crystalline substance can be regarded as cellulase crystal.

On the other hand it must be recalled that the specific activity of this crystalline preparation could be elevated only about three times as that of the starting solution, a fact which might cast some doubts upon the cellulase nature of this crystal. In view of the finding, however, of

Whitaker on *Myrothecium* cellulase that with his " highly purified preparation " only 2.4 fold purification has been achieved, the possibility still remains that the crystalline substance obtained in the present experiment might not be far from pure. It appears rather probable that cellulases are enzymes with low catalytic efficiency in contrast to those having high turnover number such as catalase and others.

This cellulase preparation was found to be active not only upon CMC, but also upon *p*-nitrophenyl β-cellobioside. Since the specific activity on the latter substance did not alter by recrystallization,* it appears certain that the same enzyme which attacks CMC may be responsible for the hydrolysis of this cellobioside. In this respect special attention deserves to be paid to the fact that both β-glucopyranoside bonds of *p*-nitrophenyl β-cellobioside, namely agluconic and holosidic bond, are hydrolyzed by the purified *Irpex* cellulase. As this preparation was free of cellobiase the cleavage of the holosidic bond of this substrate may be ascribed to the action of an enzyme other than cellobiase.

In view of the findings that phenyl α-maltoside withstands the action of the amylases of pancreas and of *Aspergillus oryzae* and that maltotriose is resistant to malt β-amylase, it seems of special interest that *Irpex* cellulase hydrolyzes besides CMC also aryl β-cellobioside.

SUMMARY

1. A crystalline substance having relatively high cellulase activity was obtained from the culture solution of *Irpex lacteus* by fractionation with acetone. The specific activity remained unchanged by recrystallization, suggesting that the crystal itself might be regarded as cellulase.

2. The crystalline preparation was found to split besides CMC also *p*-nitrophenyl β-cellobioside both at agluconic and holosidic bond, although the enzyme preparation was completely free of cellobiase.

3. The rate of the enzymic hydrolysis of the holosidic bond of *p*-nitrophenyl β-cellobioside fell rapidly with time. On the basis of inhibition experiments this phenomenon was interpreted in terms of a specific inhibition of cellulase by the split products such as glucose, cellobiose and β-glucoside. Phenol showed no inhibitory action upon the hydrolysis of this bond.

The writer wishes to express his deep gratitude to Dr. T. Miwa, Professor in the

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* Unpublished observation.

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BIOSYNTHESIS OF THREONINE FROM HOMOSERINE

IV. FRACTIONATION OF THE ENZYME SYSTEM AND OCCURRENCE OF AN INTERMEDIATE[1]

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Previous studies on the mechanism of threonine biosynthesis (*1*, *2*) have shown that a soluble enzyme system, prepared from acetone-dried yeast, catalyzed L-threonine synthesis from L-homoserine in the presence of adenosinetriphosphate (ATP) and Mg^{++}. Cohen *et al.* (*3*, *4*, *5*), also investigating threonine synthesis with *E. coli* extract, suggested that at least one intermediate occurs between homoserine and threonine. However, for lack of direct evidence, the mechanism of the reaction, especially the exact point at which ATP functions, has remained unknown.

Recent studies in our laboratory[2] have demonstrated that the yeast enzyme system, catalyzing threonine synthesis from homoserine, was separated into two fractions, and the over-all reaction was completed only in the presence of both fractions. On the basis of experimental results obtained with separate enzymes, it has become apparent that the reaction leading to threonine synthesis from homoserine involves two distinct steps. In the first step, a stable intermediary product is formed from homoserine and ATP by a separate enzyme, and then it was converted to threonine by the another.

The present paper is concerned with the separation of these two enzymes, and with the results obtained thus far in studies of the mechanism of the reaction.

METHODS AND MATERIALS

Enzyme activity was followed by the rate of threonine synthesis with homoserine

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2) Preliminary report of some of this work was already published ; *J. Agr. Chem. Soc. Japan*, **29**, 658 (1955)

as a substrate. Usually the reaction mixture contained 20 μM of DL-homoserine, 5 μM of ATP, 10 μM of magnesium sulfate, 100 μM of potassium phosphate, pH 6.8, 20 μM of sodium fluoride and the enzyme solution in a total volume of 2.0 ml. After incubated at 37°, the reaction was stopped by heating in a boiling water bath for 5 minutes, cooled, and centrifuged. Threonine in 0.5 ml. of the supernatant was estimated colorimetrically as described previously (*2*). Variations in the enzyme assay system will be noted in each table. As a blank test, the same procedure was always carried out without added homoserine.

Baker's yeast was obtained commercially. DL-Homoserine and barium salt of ATP were prepared as in the previous work (*2*). Sodium salt of ATP was prepared from barium salt just before use, and was determined spectrophotometrically or by the liberation of acid-labile phosphate. Protein was determined by Kjeldahl method.

RESULTS AND DISCUSSION

I. Fractionation of Enzymes

Extraction—Crude extract of yeast was prepared by essentially the same method described in the previous paper (*2*). 15 g. of acetone-powder, broken into fine particles, was extracted with 5 volumes of potassium phosphate buffer for 30 hours at 3°. Insoluble materials were centrifuged at $10^4 \times g$ for 30 minutes at 0° and reextracted with 1.5 volumes of the same buffer. The combined supernatant contained 3.3 mg. of protein nitrogen per ml. Specific activity, 149 ; total units, 34,440. A unit of activity has been arbitrarily defined as 0.01 μM of the threonine synthesized in an hour at 37° per 2.0 ml. of reaction mixture under the usual assay condition ; specific activity, units of activity per mg. of protein-nitrogen.

Acid Treatment—The pH of the crude extract, now about pH 6.8, was adjusted to 4.8 with 1 *N* HCl at 0°, and the inactive precipitate was centrifuged. The supernatant contained 1.2 mg. of protein-nitrogen per ml. Specific activity, 213 ; total units, 16,320.

Fractionation with Ammonium Sulfate—The supernatant solution from acid precipitation was adjusted to pH 6.8 with 1 *N* potassium carbonate. It was then fractionated with solid ammonium sulfate at −5°, pH 6.6, into three fractions by the successive addition of the salt ; *Fraction I* between 0 and 0.45, *Fraction II* between 0.45 and 0.65, and *Fraction III* between 0.65 and 1.00 ammonium sulfate saturation. In each step the salt was added over a half hour interval and mechanical stirring was continued for a total of 2 hours before centrifuging at $10^4 \times g$ for

30 minutes at 0°[3]. *Fractions I* and *II* were dissolved in each 15 ml. of 0.1 *M* potassium phosphate buffer, pH 6.8, and *Fraction III* was dissolved in 5 ml. of the buffer, and then, they were subjected to assays of the enzyme activity. The results of a typical fractionation are illustrated in Table I. While each fraction showed very low activity

TABLE I

Fractionation of Yeast Extract and Enzymatic Activities of the Fractions

Fraction No.	$(NH_4)_2SO_4$ saturation	Total protein-N	Fractions combined						
	per cent	*μg.*							
I	0- 45	11.9	+			+		+	+
II	45- 65	31.8		+		+	+		+
III	65-100	4.8			+		+	+	+
Threonine formed μM			0.04	0.17	0.05	0.87	0.19	0.18	0.95
Units per mg. of protein-N			20	32	21	119	25	41	98

The reaction mixtures contained, expressed as micromoles per 2.0 ml., 5 ATP, 20 DL-homoserine, 10 magnesium sulfate, 100 potassium phosphate, 20 sodium fluoride and the enzyme with 130 to 500 μg. of protein-nitrogen. Incubated for an hour at 37°. Threonine formed was expressed as micromoles per 2.0 ml. of the reaction mixture.

when assayed separately, the combination of *Fractions I* and *II* resulted in a much larger increase in activity than would be expected from mere summation. Unfortunately, the present fractionating procedure of the enzyme system caused a large loss of activity.

Fraction II was sometimes found to have a small activity which is undoubtedly due to slight contamination with the other. The mutual contamination proved to be completely removed by a second precipitation, as is shown in Table II. *Fraction I A* was obtained by refractionation of *Fraction I* and represents the fraction again precipitating at 0 to 0.4 saturation. *Fractions II A* and *II B* were obtained by refractionation of *Fraction II* and represent the fractions precipitating at 0.45 to 0.5 and 0.5 to 0.65 saturation, respectively. Since this was carried out

3) Precipitate was not completely removed by the centrifugation from the mixture saturated with the salt.

TABLE II

Reprecipitation of Two Fractions and Enzymatic Activities of the Fractions

Fraction No.	$(NH_4)_2SO_4$ saturation	Total protein-N	Fractions combined						
	per cent	*μg.*							
IA	0–40	8.1	+			+		+	+
IIA	45–50	10.4		+			+	+	+
IIB	50–65	15·6			+	+	+		+
Threonine formed μM			0	0.07	0	1.16	0.40	0.16	1.10
Units per mg. of protein-N			0	20	0	162	46	33	110

Fraction IA was obtained by refractionation of *Fraction I*, and *Fractions IIA* and *IIB* by refractionation of *Fraction II*. Conditions as described for Table I.

without dialysis, the salt concentrations given represent the amount added rather than those actually present. The precipitates were dissolved in a minimum amount of 0.1 *M* potassium phosphate buffer, dialyzed overnight against distilled water at 2°, then a small precipitate formed was discarded by centrifugation. The clear supernatants were employed as enzyme solutions. Enzymes may be lyophilized at any stage of fractionation and are stable in this form at room temperature for at least a month.

Since *Fraction I A* is associated with the enzyme which catalyzes the first step of the over-all reaction, as is shown in the latter part of this paper, it will be hereafter designated as *Enzyme I*, and *Fraction II B* as *Enzyme II*. Both *Enzmes I* and *II* must be present together for threonine synthesis from homoserine. When one of the enzymes is in excess, the rate of the threonine synthesis is proportional to the amount of the other enzyme present. This is shown in Fig. 1. Curve 1 gives the amount of the threonine synthesized in a standard test system as a function of the concentration of *Enzyme I* in the presence of an excess of *Enzyme II*; Curve 2, as a function of the concentration of *Enzyme II* in the presence of an excess of *Enzyme I*. Further purification and properties of both enzymes will be reported in a following paper.

II. Mechanism of Threonine Synthesis from Homoserine

In order to elucidate the reactions in which each enzyme par-

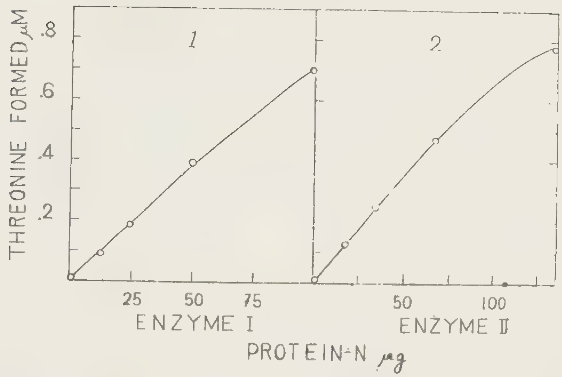

FIG. 1. Threonine formation as a function of the concentrations of *Enzme I* and *II*.

The reaction mixtures of Curve 1 contained, expressed as micromoles per 2.0 ml., 20 DL-homoserine, 5 ATP, 10 magnesium sulfate, 20 sodium fluoride, 100 potassium phosphate, *Enzyme II* with 566 μg. of protein-nitrogen and various amounts of *Enzyme I*. The reaction mixtures of Curve 2 differed from those of Curve 1 in that they contained *Enzyme I* with 204 μg. of protein-N and varying amounts of *Enzyme II*. Incubated for an hour at 37°. This experiment was carried out with lyophilized enzyme preparations.

ticipates, further studies were attempted concerning the occurrence of an intermediate between homoserine and threonine, and the order of the reaction sequence. The experimental procedures were of the following character: 1) Homoserine and ATP were incubated initially with *Enzyme I* in the presence of Mg^{++} for 30 minutes, then *Enzyme II* was added to the reaction mixture and incubation was continued for additional 30 minutes. At the end of the reaction, the solution was deproteinized by heating and aliquots were taken for the estimation of threonine formed ; 2) On the contrary, substrates were initially incubated wih *Enzyme II* and subsequently with *Enzyme I* for an additional incubation period ; 3) Incubation was conducted in a standard assay system with both enzymes for 30 minutes ; 4) The procedure is the same as for 3), except 60 minutes incubation instead of 30 minutes.

These experimental series were compared in Table III, with respect to the amount of threonine formed. In the first system, the amount of threonine sysntheized was twice as much as in the second or third, and was comparable to that of the fourth system which was incubated with

TABLE III

Threonine Synthesis by Separate Enzymes (1)

Exptl. No.	First incubation		Second incubation		Threonine formed
	Conditions	Time	Additions	Time	
		min.		*min.*	μM
1	*Enzyme II* omitted	30	*Enzyme II*	30	0.40
2	*Enzyme I* omitted	30	*Enzyme I*	30	0.22
3	Complete	30	—	—	0.28
4	”	60	—	—	0.61

Complete system contained, expressed as micromoles, 20 DL-homoserine, 5 ATP, 10 magnesium sulfate, 20 sodium fluoride, 100 potassium phosphate, pH 6.8, 0.25 ml. of *Enzme I* with 360 μg. of protein-nitrogen and 0.25 ml. of *Enzyme II* with 330 μg. of protein-nitrogen in a final volume of 2.0 ml. Initial reaction mixtures of experimental No. 1 and No. 2 differed from those of complete system, besides the omission of one enzyme, in that they contained 75 μM of potassium phosphate in a total volume of 1.75 ml. At the end of the first incubation, they were brought to the same condition as in the complete system by the addition of omitted enzyme. In these experiments, lyophilized enzymes were employed.

both enzymes for 60 minutes. It may be suggested, therefore, that a two-step reaction is operating and that, with *Enzyme I*, the formation of an intermediate precursor for threonine would take place initially, and the product was transformed into threonine by the function of *Enzyme II*. An alternative hypothesis, that a conversion of homoserine to threonine occurs at one step in the simultaneous presence of both enzymes, appears to be less likely from the above results. Moreover, this conception was supported by the following experiments.

Since both enzymes were found to be rather labile to thermal treatment, *i.e.*, they were inactivated completely when their solutions were kept at 70° for 5 minutes, advantage has been taken of this behavior in studying more detailed mechanism. At the end of the preliminary incubation, reaction mixtures were kept in a water bath at 70° for 5 minutes in order to inactivate the enzyme initially added, and then another enzyme or a supplemental substance were added for an additional incubation period. The results are shown in Table IV. When homoserine was preliminary incubated with *Enzyme I* in the presence

TABLE IV

Threonine Synthesis by Separate Enzymes (2)

Exptl. No.	First incubation		Second incubation		Threonine formed
	Conditions	Time	Additions	Time	
		min.		*min.*	μM
1	*Enzyme II* omitted	30	*Enzyme II*	60	0.59
2	"	60	"	60	0.76
3	*Enzyme I* omitted	60	*Enzyme I*	60	0.02*
4	*Enzyme II* and ATP omitted	60	*Enzyme II* and ATP	60	0.01*
5	*Enzyme II* and homoserine omitted	60	*Enzyne II* and homoserine	60	0.03*

Incubation procedure was the same as in Table III with the exception of thermal inactivation of the enzyme at the end of the first incubation. Omission of either homoserine or ATP was accompanied by the decrease of 0.5 ml. of the volume of reaction mixture. Omission of an enzyme also caused the decrease of 25 μM of potassium phosphate and 0.25 ml. of the volume of reaction mixture. At the beginning of the second incubation, composition of all systems become uniform, except that an enzyme initially added was inactivated.

* These values are not analytically reliable.

of ATP and Mg^{++}, threonine was synthesized by the subsequent prolonged incubation with *Enzyme II*, even if *Enzyme I* was inactivated before the addition of *Enzyme II*. Whereas, in the case of the incubation initially with *Enzyme II* and subsequently with *Enzyme I*, threonine formation was not observed. If the preliminary incubation with *Enzyme I*, followed by heat-inactivation, was carried out in the absence of either homoserine or ATP, threonine was not also synthesized by the additional incubation with *Enzyme II*. These results indicate that a rather stable intermediate is formed from homoserine, ATP and *Enzyme I*, then the product acts as a specific substrate for *Enzyme II* and resulted in the formation of threonine. In the light of this information the reactions catalyzed by *Enzymes I* and *II* may be represented according to Reaction (a) and (b).

(a) $\text{Homoserine} + \text{ATP} \xrightarrow[Mg^{++},\ \textit{Enzyme I}]{} \text{intermediate (phospho-homoserine).}$

(b) $\text{Intermediate} \xrightarrow[\textit{Enzyme II}]{} \text{threonine.}$

The formation of the intermediate can be detected by paper chro-

matography with phenol-water as solvent. Its *Rf* value is 0.14 which is easily distinguished from that of homoserine, 0.58. The intermediate has been isolated as barium salt and characterized as phosphorylated homoserine[4] chiefly from the evidences that homoserine and inorganic phosphate were liberated by acid hydrolysis, and that the 2,4-dinitrophenyl (DNP)-derivative of the intermediate was identified with synthetic N-DNP-*O*-phospho-homoserine. The procedures of isolation and indentification will be described in a forthcoming paper. When an isolated intermediate was used as substrate, neither ATP nor Mg^{++} was required in Reaction (b). The requirement for Mg^{++} in the over-all reaction, therefore, may be accounted by the participation of it in Reaction (a). This is also compatible with the mechanism of most ATP-dependent reactions.

SUMMARY

Two enzymes which catalyse threonine synthesis from homoserine have been separated from Baker's yeast extract by ammonium sulfate fractionation. It has become apparent that two distinct reactions are involved in the over-all reaction. With *Enzyme I*, associated with the fraction precipitating at lower salt concentration, a stable precursor for threonine is synthesized from homoserine and ATP in the presence of Mg^{++}, then the product acts as specific substrate for *Enzyme II*, which is associated with the fraction precipitating at higher concentration of the salt, resulting in the formation of threonine even in the absence of ATP.

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4) *Enzyme I* may be termed *homoserinekinase* from the enzymatic mechanism of the Reaction (a).

NOTE

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A SIMPLIFIED METHOD FOR THE PREPARATION OF FLAVIN-ADENINE DINUCLEOTIDE BY CIRCULAR PAPER CHROMATOGRAPHY

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The senior author (*1*) reported the preparation of flavin-adenine dinucleotide (FAD) from hog liver by using a filter paper chromatopile. By the same principle as this method, a circular filter paper chromatography may be applied to the praparation of FAD. Since *Eremothecium ashbyii* was proved to be a good source of FAD (*2*), the fungus was used as the starting material. Application of the circular paper chromatography to the extract of this fungus made the preparation of FAD more simple and easy, which can be used satisfactorily for laboratory work. The method is described in this paper.

Material, Apparatus, and Assay Method

Eremothecium ashbyii—From the stock culture, 1 platinum ear of the spores of the fungus was put into the medium and grown by a shaking culture for 72 hours at 30°. One ml. of the cultivated medium was transfered into 500 ml. of the medium, and again cultivated under the same conditions.

The medium was of the following composition: 1.0 g. of glucose, 0.5 g. of peptone, 0.2 g. of KH_2PO_4, 0.1 g. of $MgSO_4 \cdot 7H_2O$, 0.1 g. of NaCl were dissolved in water and made up to 100 ml. The shaking frequency was regulated to 120 per minute.

After the cultivation, 20 g. in wet weight of the fungus containing 30 mg. of FAD and 29 mg. of free riboflavin was obtained from the medium by centrifugation.

Apparatus for Circular Paper Chromatography—Vacuum desiccator (30 cm. in diameter) was used as the apparatus, as reported by Töppel (*3*). A sheet of thick circular filter paper (Toyo Roshi No. 26 H) was held between the body and the cover of a desiccator. A glass tube having a solvent pool was thrust through a rubber stopper at the top of the cover of the desiccator and its tip touched the filter paper as shown in Fig. 1.

Assay of Flavin Compounds—The separating determination of riboflavin nucleotides

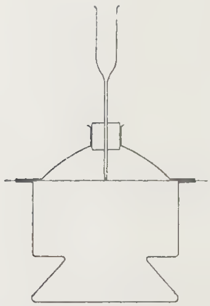

FIG. 1. Apparatus for circular filter paper chromatography.

was made by paper electrophoresis reported by the authors (*4*), and the purity of FAD was estimated by measurement of the light absorption at 450 mμ using $\varepsilon = 11.3 \times 10^3$ L. mole^{-1}. cm^{-1}. (*5*).

Procedure

Extraction of Flavins—Twenty grams of the wet mass of fungus described above was extracted with 60 ml. of water at 70–80° for 15 minutes and the residue separated by centrifugation was extracted again. The combined aqueous extract was condensed to a syrup under reduced pressure and put into 100 ml. of ethanol with violent stirring. The amorphous yellow precipitate deposited in the yellow ethanolic solution was separated by filtration through a Buchner funnel and extracted with 40 ml. of liquid phenol. A clear dark brown-colored phenol solution was obtained by centrifugation. Five volumes of ether was added to the phenol solution and the mixture was extracted repeatedly with a small quantity of water. Thus, 30 ml. of an aqueous solution of flavins was obtained, containing 23 mg. of FAD and 11 mg. of free riboflavin. A larger part of protein and free riboflavin were removed by the procedure described above.

Application of Circular Paper Chromatography—The aqueous solution of flavin mixture was concentrated to a few ml. volume under reduced pressure, adsorbed on about 0.5 g. of powdered cellulose and dried *in vacuo*. The dark brown powder was placed on top of a powdered cellulose layer filled in the lower part of the glass tube, and the tip of the tube was made to contact with the center of the filter paper. Then, the solvent (upper layer of the mixture of 4 volumes of *n*-butanol, 1 volume of acetic acid, and 5 volumes of water) was filled in the solvent pool of the glass tube, and developed for 30 hours. The circular band of free riboflavin shifted to the outer edge of the filter paper, and the band of FAD remained near the center of the filter paper, diameter of circle being about 14 cm. Flavin mononucleotide was not found in the fungus.

The FAD zone of the paper was cut out, washed with ether, and cut into small

pieces. FAD contained in the filter paper was extracted by heating it in water at 70° for 20 minutes.

The extract contained 20.3 mg. of FAD. The purity of it was 18.3 per cent and the absorption ratio E_{260}/E_{450} was 4.0 at this stage of the procedure.

Further Purification—The aqueous extract of FAD from the filter paper was purified by using " Florisil," as reported by Dimant and others (*6*). The extract was adsorbed on a column of " Florisil," which was washed with 2 per cent acetic acid and then with water. After the layer of adsorbed FAD reached the lower portion of the tube by eluting it with 0.5 per cent aqueous pyridine solution, FAD was eluted with 5 per cent pyridine solution. Since FAD adsorbed on " Florisil " for a long time converted to fourth flavin compound (*7*), the above adsorption procedure must be carried out as quickly as possible.

Pyridine in the eluate was removed by extraction with chloroform at pH 8.0, the pyridine-free FAD solution was saturated with ammonium sulpahte, and extracted with liquid phenol. The phenol layer was separated by centrifugation, added with one-half volume of benzyl alcohol and dried by adding anhydrous sodium sulphate. After the phenol-benzyl alcohol mixture was filtered, five volumes of ether was added, and FAD was extracted with a small quantity of water.

The FAD solution was poured into absolute ethanol and the precipitated yellow solid was dried *in vacuo*.

In this powder, 17.6 mg. of FAD was found (yield, 58.5 per cent). The absorption spectrum of this FAD powder was similar with that of predecessor's report (*5*), and the absorption ratio E_{260}/E_{450} was 3.475. The purity of this FAD powder was 43.3 per cent.

SUMMARY

A circular paper chromatography was applied for the purification of FAD contained in the extract of *Eremothecium ashbyii*. By relatively simple procedures, FAD powder was obtained in an yield of 58.5 per cent and the purity was 43.3 per cent.

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